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Dissertation

Dynamic of allelopathically active polyphenolic substances of *Myriophyllum verticillatum* L. and factors influencing allelopathic effects on phytoplankton

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NADINE BAUER

Dekan: Prof. Dr. Andreas Herrmann

Gutachter: 1. Prof. Dr. R. Ehwald

2. PD Dr. S. Hilt

3. PD Dr. N. Kamjunke

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CONTENT/ INHALTSVERZEICHNIS

ABSTRACT

Dissolved organic compounds released by macrophytes can have allelopathic effects on phytoplankton and thereby contribute to stabilize the clear water state of shallow lakes. To investigate influencing factors on allelopathic effects on phytoplankton this thesis tested the following hypotheses 1) that the temporal dynamic of allelochemicals in the plant (*Myriopyllum verticillatum*) is related to the nutrient status of the plant and congruent with the dynamic of growth response of phytoplankton 2) that solar radiation and bacteria influence the allelochemical and 3) that the presence and the composition of the bacterial community influence algal response to the allelochemical.

Total phenolic compounds (TPC) of apicals of *Myriophyllum verticillatum* L. ranged between 38 to 122 mg TPC g^{-1} DW during four years (2004-2007) and its dynamic corresponded to growth inhibition of *Anabaena variabilis*. Nutrient content partly explained the TPC dynamics. TPC was positively correlated with C/N ratio for all but one year and negatively correlated with total Phosphorus in two of four years. Highest amounts of TPC in plant tissue were found from May to July. Also the maximal concentrations of single polyphenolic compounds with mayor allelopathic activity in bioassays were detected in May, when macrophytes compete with algae for light to grow to the water surface. These phenolic compounds were identified as isomers of Hexahydroxydiphenoyl -di- and -tri-galloylglucose by HPLC and LC-MS structurally similar to the polyphenolic model substance tannic acid (TA).

Bacteria and solar radiation were found to influence the diagenesis of the allelochemical and its effect on phytoplankton. Photolytic and microbial TA degradation formed recalcitrant humic substances like complexes and easy degradable low molecular weight substances. The photolytic recalcitrant degradation products resulted in lower algal growth than respective products formed in the dark. Phytoplankton response to TA was species specifically different depending on temperature and the presence or absence of bacteria. Phytoplankton-bacteria interaction, phytoplankton start concentration and bacterial community composition were found to be important for quantitative and qualitative (positive or negative) phytoplankton response to TA. Bacteria of the genus Pseudomonas were isolated that were able to degrade TA, thus lowering effects of this substance on algal growth. A compare of different approaches performed to test algal sensitivity to allelochemicals (*in situ* with dialysis bags, in coexistence experiments in aquaria and *in vitro* in reagent tubes) revealed qualitatively comparable results for fluorescence and deviating results for particle based growth parameters.

By the use of analytical and molecularbiological methods the factors identified to regulate allelopathy were the temporal dynamics of allelochemicals in the plant, photolytic and bacterial modification of the diagenesis of the allelochemical such as degradation processes and temperature dependent species specific algal sensitivity and changes in mutualistic interaction of bacteria and phytoplankton.

Keywords: allelopathy, bacteria-phytoplankton interaction, *Myriophyllum verticillatum*, polyphenols, tannic acid

ZUSAMMENFASSUNG

Durch die Freisetzung von allelopathisch aktiven Substanzen können Makrophyten das Wachstum von Phytoplankton beeinflussen und damit den Klarwasserzustand von Flachseen stabilisieren. Inwiefern die Wirkung von Allelochemikalien im Gewässer auf das Phytoplankton z.B. durch Umweltfaktoren beeinflusst wird, ist bisher unerforscht und Gegenstand dieser Arbeit. Dabei werden die folgenden Hypothesen getestet: 1) dass der allelopathische Effekt auf das Phytoplankton mit der zeitlichen Dynamik polyphenolischer Substanzen in der Pflanze korreliert und vom Nährstoffgehalt der Pflanze abhängt, 2) dass Sonnenlicht und Bakterien Allelochemikalien und ihre Wirksamkeit beeinflussen und 3) dass die Zusammensetzung der Bakteriengemeinschaft die allelopathische Wirkung auf das Phytoplankton beeinflussen kann. Die Untersuchung der zeitlichen Dynamik des Gesamtphenolgehaltes im Apikalmeristem von *Myriophyllum verticillatum* L zeigte Schwankungen zwischen 38 - 122 mg g-1 Trockenmasse über einen Zeitraum von vier Jahren 2004-2007 und korrelierte mit der Wachstumshemmung von *Anabaena variabilis*. Die hohe Dynamik des Gesamtphenolgehaltes war teilweise durch den Nährstoffgehalt in der Pflanze erklärbar. So korrelierte der Gehalt polyphenolischer Stoffe positiv mit dem C/N Verhältnis (in drei der vier Jahre) und negativ mit dem Gesamtphosphatgehalt der Pflanze (in zwei der vier Jahre). Ein generell höherer Gesamtphenolgehalt in der Trockenmasse der Pflanze sowie der hauptaktiven Fraktionen polyphenolischer Substanzen im Biotest wurde im Mai bis Juli gefunden, wenn *M. verticillatum* zur Wasseroberfläche wächst und mit dem Phytoplankton um Licht konkurriert. In den hauptaktiven Fraktionen der Pflanzenextrakte wurden mittels HPLC und MS Isomere von Hexahydroxydiphenoyl di- und -tri-galloylglucose – in Struktur ähnlich der Modellsubstanz Tanninsäure (TA) identifiziert. Abiotische und biotische Einflüsse der Umwelt auf die Allelochemikalie z.B. Bakterien und Sonnenlicht führten bei der Modellsubstanz TA neben schnell abbaubaren niedermolekularen Stoffen zur Bildung von refraktären hochmolekularen Verbindungen u.a. Huminstoffe. Diese photolytischen Umwandlungsprodukte bewirkten ein geringeres Algenwachstum - verglichen mit Parallelkulturen, die mit im Dunkeln abgebauter TA inkubiert wurden. Temperatur beeinflusste artspezifisch die Reaktion der untersuchten Algen auf TA. Dabei waren die Art und das Ausmaß der Reaktion von der Anwesenheit von Bakterien abhängig. Die Algenanfangskonzentration, Phytoplankton-Bakterien Interaktionen und die Zusammensetzung der Bakteriengemeinschaft beeinflussten sowohl positiv als auch negativ die allelopathische Wirkung der Allelochemikalie auf die Alge. Es konnten Bakterien der

Gattung Pseudomonas isoliert werden, die in der Lage waren, TA abzubauen und deren allelopathische Effekte zu mindern.

Schlagworte: Allelopathie, Bakterien-Phytoplankton Interaktionen, *Myriophyllum verticillatum*, Polyphenole, Tannninsäure

1 INTRODUCTION

Allelopathy has been controversially discussed mainly due to its difficulties to poof allelopathic effects on ecosystem level (Gross et al. 2007). One major problem is the lack of easy accessible chemical methods to track and identify the exuded biochemically active allelopathic substances in the water. Physical, biotic and chemical processes occur concurrently and interfere with the allelopathic effects. Their effects on the target organisms are hard to be traced back exclusively on allelopathic activity (Harper 1977) and seldom all criteria proposed by Willis (1985) can be fulfilled to demonstrate allelopathy (Legrand 2003). In the past years, increasing reports of allelopathy confirm its existence and its structuring effect on primary producers in freshwater ecosystems (Gross 2003a; Inderjit und Dakshini 1994; Leao et al. 2009). However, the evaluation of its ecological relevance is hindered by the interference of competitive processes with allelopathy in the natural ecosystem. Therefore, it is necessary to unravel factors influencing allelopathic effects in the aquatic ecosystem.

The impact of allelopathic effects will be related to the production and subsequently the content of allelochemicals in the donor plant and factors changing the allelochemical after release into the aquatic environment or the allelopathic effect on the phytoplankton. In this thesis the general concept of ‘donor organisms (i.e. submersed plants)’-‘released allelochemical’-‘effect on target organisms (i.e. phytoplankton)’ is expanded by biotic and abiotic factors interfering with the allelochemical as well as with species specific response of target organisms.

1.1 Allelopathy in shallow lakes

The term “allelopathy” was established by Molisch (1937) to describe biochemical interaction between plants and microorganisms and is composed of the Greek words ‘allelon’ and ‘pathos’, which signify 'mutual' and 'affection' or 'harm'. Rice (1984) refined his earlier definition restricted to harmful interaction by one plant, including microorganisms (bacteria, fungi and microalgae), on another plant trough production of chemical compounds to both inhibitory and stimulatory effects on growth. The Allelopathy Society (1996) expanded allelopathy for secondary plant, algae, bacteria or fungi metabolites that influence the growth and development of biological and agricultural systems. Until now, most research has focused on negative effects arising by allelopathic compounds originating from plants or microorganisms. In this thesis I will include both positive and negative effects of allelopathy.

Allelopathy is a phenomenon described for many ecosystems and for all stages of succession (Rice 1984; Rice 1972; Rice 1977). Most studies focus on terrestrial ecosystems ranging from grass or weed (Inderjit 1996; Gonzalez et al. 1995) to agricultural and forest ecosystems (Sanchez-Moreiras et al. 2003; Rizvi et al. 1999). In agriculture allelopathy received attention for sustainable management of weed (Singh et al. 2003) and of biotic resources (Anaya 1999).

In aquatic ecosystems we are just beginning to understand how allelopathy affects the aquatic system compared to terrestrial allelopathy. Allelopathy in concert with other environmental factors influences phytoplankton community structure and succession and the formation of blue-green algal blooms (Rojo und Cobelas 1994; Keating 1977; Havens et al. 2001; Celewicz-Goldyn 2010). Allelopathic interactions have been described for all primary producing organisms in aquatic ecosystems, i.e. between angiosperms and macroalgae, among macrophytes, between macrophytes and epiphytes or phytoplankton (Gross 2003a; Gopal und Goel 1993; Ervin und Wetzel 2003) and among phytoplankton (Harlin 1987; Havens et al. 2001; Nan et al. 2008). Allelopathic interaction also exists between phytoplankton and macrophytes (Pflugmacher 2002) and among bacteria (Gillor et al. 2007). The present work focuses on allelopathic effects of macrophytes on phytoplankton.

Submerged macrophytes exert a multitude of ecosystem functions. They occupy key interfaces in lake ecosystems and can substantially contribute to primary productivity (Noges et al. 2010). Macrophytes physically structure aquatic ecosystems, provide habitat for epiphytes and herbivore organisms and contribute to sedimentation of particulate organic matter (Carpenter und Lodge 1986). During growth and decay macrophytes release dissolved organic matter (DOM), a by-product of photosynthesis (Findlay et al. 1992; Perez und Sommaruga 2006). These dissolved organic substances constitute a potential source of carbon for water column metabolism (Demarty und Prairie 2009) and provide a high-quality substrate for bacterial growth (Wetzel und Manny 1972). An unknown fraction of plant derived DOM consists of biochemically active allelochemicals. Allelochemicals are chemically diverse secondary plant metabolites and exert multifunctional properties of allelopathic interaction (Einhellig 1995). When released into the water allelopathic substances can inhibit growth of epiphytes and phytoplankton and thereby provide a competitive advantage for the struggle for light, because they reduce phytoplankton shading of the macrophyte.

In shallow lakes these allelopathic substances might have effects on ecosystem level if macrophytes occupy a substantial part of the littoral zone of shallow lakes. Two alternative stable

states occur in shallow lakes. The turbid state is dominated by phytoplankton, while the clear-water state is dominated by macrophytes. By the release of allelochemicals macrophytes contribute amongst other factors to the stabilization of the clear water state (Scheffer, 1989). Shifts between the two states generally occur abrupt and are difficult to reverse (Scheffer et al. 1993; Schmitt und Nixdorf 1999). Both states are maintained by positive feedback mechanisms (Fig. 1). The role of allelopathy for the resilience of this bi-stability has been discussed controversially and requires further investigation (Hargeby et al. 2004; Scheffer 1998).

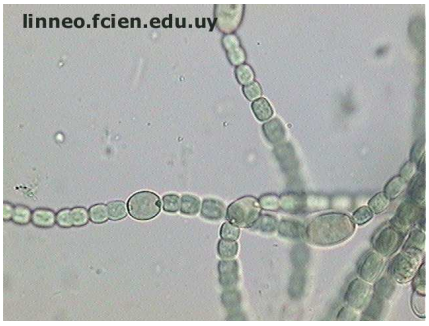


Fig. 1: Scheme of feedback mechanisms between submerged macrophytes and water turbidity in shallow lakes (Scheffer 1998). The factors addressed in this study are circled in green.

The still ongoing debate on the relevance of allelopathic effects is mainly attributed to "the lack of knowledge about the mechanisms involved in allelopathic interaction" (Ervin und Wetzel 2003). There has been increasing evidence that allelopathically active submerged macrophytes can stabilize clear-water states in shallow lakes (Hilt et al. 2006; Hilt und Gross 2008; Gross et al. 2007). However, it remains difficult if not impossible to proof allelopathic effects of macrophytes *in situ* (Gross et al. 2007) due to interference with environmental factors. Laboratory experiments can exclude the influence of interfering factors as competition or nutrient limitation, however, transferability to ecosystem level is restricted. Investigation of allelopathic impact on community level is complicated due to the high variability of phytoplankton response to allelochemicals (Körner und Nicklisch 2002; Mulderij 2005). Interfering abiotic and biotic factors that might alter the properties of allelopathic substances and/or the susceptibility of the target organism e.g. solar radiation, temperature and the bacterial influence were not investigated until now. However, degradation or inactivation of the allelopathic compounds might avert allelopathic effects.

1.2 Allelopathic substances in aquatic macrophytes

1.2.1 Allelopathic substances

Investigating the variability of allelopathic substances requires the knowledge of the chemical nature of allelopathic active compounds that exert allelopathic activity. A survey of allelopathically active macrophytes was given by Mulderij (2006). However, most studies were performed by using unidentified plant extracts, cell free filtrates or coexistence experiments. From the reviewed studies on allelopathy of submerged macrophytes on phytoplankton only a minority identified the allelopathic substances that were tested on the phytoplankton summarized in Table 1 (at the end of the *Introduction*). Out of this selection only two studies could identify the main allelopathic active substance by activity guided fractionation (Gross et al. 1996; Xian et al. 2006). No studies were found comparing the sensitivity of target organisms to allelochemicals under different abiotic and biotic conditions. This may be attributed to methodological challenges detecting and identifying the allelopathically active substances and tracing its allelopathic activity. However, the chemical structure of the allelochemical is essential for evaluating their allelopathic effect (Inderjit 1996) and to understand potential effects of abiotic and biotic factors on the allelopathic activity of allelopathic compounds.

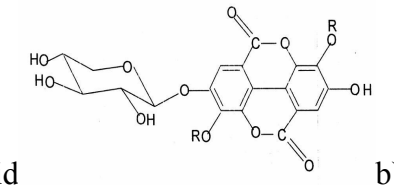
The chemical nature of most allelochemicals is characterized by amphiphilic structures, a mixture of aliphatic hydrophobic structures and hydrophilic functional groups. The production of hydrophilic and lipophilic compounds at the same time may represent an advantage for the macrophyte resulting in synergistic effects or acting against different target sites at the same time (Gross 2003a). Others identified increasing polarity of low-molecular phenolic compounds of *Schoenoplectus lacustris* being responsible for increasing phytotoxic effects on algae (D'Abrosca et al. 2006). Macías and colleagues (2008) stated that most allelopathically active compounds need to have one free hydroxyl group to be toxically active, suggesting that a certain degree of water solubility is necessary, whereas a high degree of lipophila should induce bio-stimulation. The spontaneous organization of allelochemicals is assumed to be important for surface associated activity, whereas micelle formation supports particle assemblages and thus sedimentation (Gross 2003a).

Among the indentified allelochemicals phenolic substances constitute a widespread substance class (Table 1) and will be in the focus of this work. Besides their allelopathic properties phenolics have different functions in plant physiology. For example, they act as plant defense against herbivores and pathogens (Haslam 1998), inhibit gastrointestinal microorganisms of

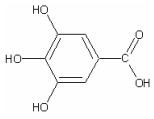
organisms consuming tannin rich food (Walenciak 2002) or serve as sunscreens such as flavonoids for protection against UV and solar radiation (Swanson 2002; Caldwell et al. 1983). In plant tissue polyphenols accumulate in cell walls, vacuoles and are associated with cell nuclei (Hutzler et al. 1998).

The genus *Myriophyllum* is known for its high allelopathic potential, which was attributed to a high content of polyphenols (Gross et al. 1996). The most allelopathically active substance from the European milfoil *Myriophyllum spicatum* could be identified as tellimagrandin II (Fig. 2), a hydrolysable polyphenol (Gross et al. 1996). The structure of allelochemicals range from low molecular weight substances (e.g. simple organic acids) to complex structures of high molecular weight (e.g. polyphenols) (Fig. 2).

Simple phenols and phenolic acids:

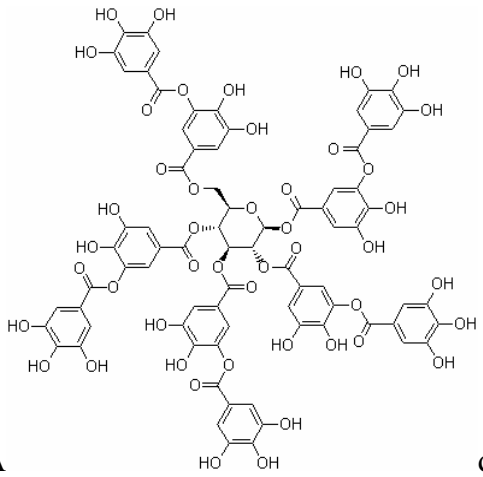


a) Ellagic acid

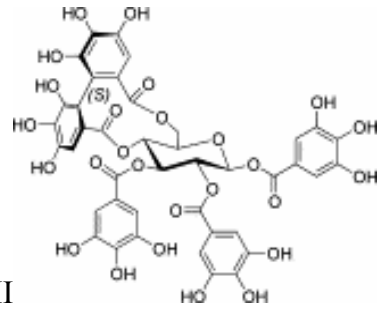


b) Gallic acid

Polyphenols:



c) TA



d) Tellimagrandin II

Fig. 2: Structure formula of allelopathic substances of *Myriophyllum spicatum* and possible polyphenolic allelopathic substances in *M. verticillatum* a) Ellagic acid, b) Gallic acid, c) Tannic acid (TA), d) Tellimagrandin II. Source: http://www.chemblink.com/products/1401-55-4.htm

About the allelopathic potential of *M. verticillatum*, a close relative of *M. spicatum*, less information is available. Aliotta (1992) suggested phenylpropanoids and fatty acids being responsible for its allelopathic activity, while (Lutz 2004) identified tannic acid and its first partitioning products gallic acid and ellagic acids as possible candidates (Fig. 2)

1.2.2 Modes of action

Allelochemicals affect many processes, two important ones are photosynthesis and enzyme activity (Einhellig 1995; Einhellig und Reigosa 2002; Gross 2003a). Inhibition of photosynthesis is an effective strategy to inhibit the growth of competing primary producers and was reported for allelochemicals from macrophytes and cyanobacteria (Einhellig 1995; Smith und Doan 1999; Leu et al. 2002). The most allelopathically active compound in *M. spicatum*, tellimagrandin II, and lipohile extracts were found to inhibit the photosystem II (PSII) by interfering with the electron transfer of *Anabaena* sp. PCC7120 (Leu et al. 2002). Tellimagrandin II and other polyphenols in *M. spicatum* are assumed to cause a higher redox midpoint potential for the non heme iron, located between the primary and the secondary quinine electron acceptors, Q_A and Q_B in the PSII (Fig. 3). These allelochemicals act on a different site of action than synthetic herbicides which affect the Q_B-binding site (Ohad und Hirschberg 1990; Huppatz 1996).

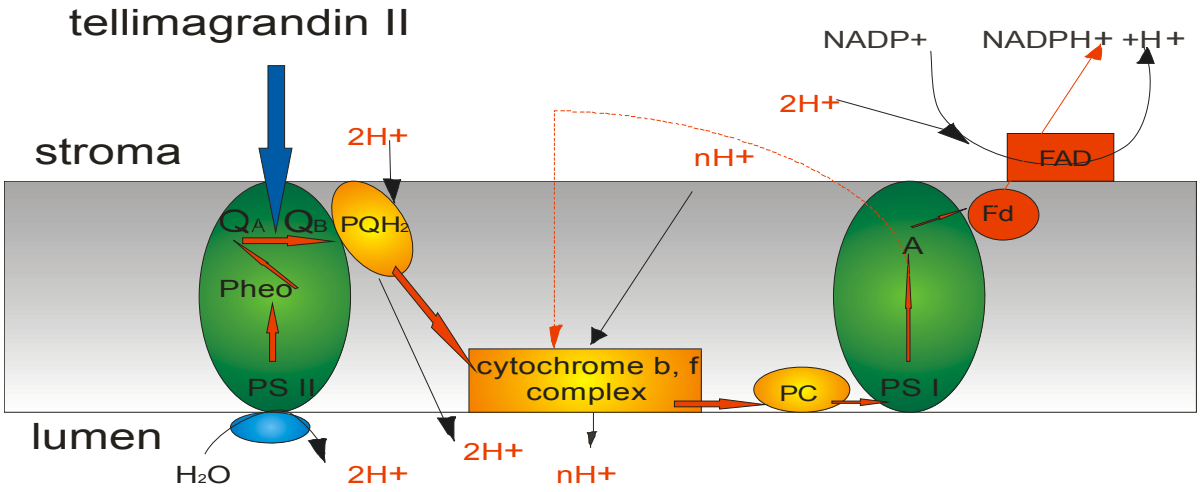


Fig. 3: Z-scheme of electron transport chain (red arrows) of the thylakoid membrane (grey bar) in the chloroplasts with photosystem I and II (green ovals, PSI and PSII) and the target site of tellimagrandin II (blue arrow) between the quinine electron acceptors Q_A and Q_B (Leu et al. 2002). Abbreviations: Pheo: pheopyhtin a (chlorophyll a), PQ: plastochinon, PQH_2: reduced plastohydrochinon, PC: phycocyanin, A: primary electrone acceptor of PS I, Fd: ferredoxin, FAD: Ferredoxin-NADP-reductase. Modified scheme, source: http://www.fsbio-hannover.de/oftheweek/120.htm

There are several enzymatic processes that can be inhibited by allelochemicals. Inhibition of enzymes is very likely for organisms producing extracellular enzymes for substrate allocation or to colonize surfaces (Wetzel und Chróst 1991). Microalgal exo-enzymes were already identified as target of allelochemicals in the first mechanistic studies on allelopathy of Rice (Rice

1977). For example, polyphenolic plant extracts from *M. spicatum* and *M. verticillatum* inhibit the enzyme alkaline phosphatase (Gross et al. 1996; Blaschke 2007). Enzyme inhibition can harm the whole organisms such as decreased dehydrogenase activity in *M. aeruginosa* resulting in increased level of reactive oxygen species (ROS) (Hong et al. 2008). This in turn will result in increased oxidative damage of cellular structures such as cell membranes. For instance, lowered activities of antioxidant enzymes, such as superoxide dismutase and peroxidase, can lead to increased oxidative damage, metal ion leakage and changes in plasma integrity (Li und Hu 2005).

Membrane perturbations is another mode of action already suggested to be a common starting point for effects of allelopathically active phenolic acids in terrestrial ecosystems (Einhellig und Reigosa 2002) and might be widespread in aquatic ecosystems, too. Fatty acids for example ethyl 2-methylacetoacetate from *Phragmites communis* were shown to be involved in changes in plasma membrane integrity and leakage of ions in the protoplast (Li und Hu 2005). Other modes of action of allelochemicals among phytoplankton are for example cellular paralysis reported of *Anabaena flos-aquae* affecting motile green algae (Kearns und Hunter 2001) as well as the inhibition of nucleic acid synthesis (Einhellig und Reigosa 2002) or the RNA polymerase by alkaloids (Doan et al. 2000).

1.2.3 Abiotic and biotic factors influencing allelochemical dynamics

Important factors influencing the extent of allelopathic effects (Ervin und Wetzel 2003) are light and nutrient limitation of both the donor organisms (Rengefors and Legrand 2001) or the target organisms (Graneli und Johansson 2003). The production of secondary metabolites for plant defense was shown to be related to light and nutrient availability (Cronin und Lodge 2003), temperature (Albert et al. 2009) and biotic factors such as herbivory (Lemoine et al. 2009) or stress (Karl et al. 2008). Allelopathy and other processes for plant defense cannot always be separated. Most secondary plant metabolites are produced for other purposes than allelopathy, for instance, for protection against solar radiation such as anthocyane production under UV exposure (Guo et al. 2008) or defense against herbivory (Karban und Myers 1989; Berenbaum 1995; Lemoine et al. 2009). Subsequently these chemicals can be released into the water and may exert allelopathic effects. Only few studies relate allelochemical production of macrophytes to abiotic and biotic influences. Gross and colleagues tested if light and nitrogen have an impact on the production and release of the allelochemical in the submerged macro-

phyte *Myriophyllum spicatum* (Gross 2003b). They found that nitrogen availability did not influence the total content of tissue-bound polyphenols, however, increased the tissue concentration of tellimagrandin II, the allelopathically most active compound, at low nitrogen and low light availability. These experiments were conducted using axenic cultures in a controlled environment to exclude the effects of additional factors such as herbivory or shading by epiphytes. Transferring the results of experimental studies to field situations remains difficult. In field manipulation experiments with *Potamogeton amplifolius* and *Nuphar avena* leaf-phenolics were significantly increased by high light and by high nutrient availability (Cronin und Lodge 2003). However, manipulation could have caused stress and thereby might have induced the production of defense substances. For terrestrial ecosystems it was argued that environmental stress (low levels of nutrients, light, and temperature) might either increase allelopathic production or the sensitivity of target organisms (Reigosa et al. 1999). Chemical defenses were found to be probably constitutive in *Elodea* and induced in *Myriophyllum spicatum* when grazed by snails (Lemoine et al. 2009).

Allelopathic compounds may be produced either on demand or represent accumulated intermediate metabolites if for example depletion of N and P availability impede further metabolic synthesis (Wright et al. 2010). Until recently, the carbon-nutrient balance hypothesis (Bryant et al. 1983) was the most influential theory predicting variation in the content of secondary plant metabolites. It states that environmental conditions that increase the carbon/nutrient ratio, e.g. high light and low nutrient availability, will result in an increased production of carbon-based secondary compounds (CBSC).

Environmental abiotic and biotic influences on the production of allelopathic compounds in macrophytes and the multi-functionality of allelopathic compounds may indicate a rather complex pattern of seasonal dynamics of allelochemicals. Until now, the highest annual tissue concentration and excretion rate of polyphenolic compounds for *Myriophyllum verticillatum* were reported in spring in a dystrophic lake (Hilt et al. 2006) and in summer for *M. spicatum* in Lower Lake Konstanz (Gross, unpublished results cited in (Hilt und Gross 2008)). In spring to summer submerged macrophytes shoots grow upwards in the water column to reach the water surface. Macrophytes that exude allelochemicals can inhibit epiphytic cover and thereby reduce competition for light with phytoplankton until they grow to the surface and subsequently form dense stands of macrophyte and then limit phytoplankton growth by shading.

1.3 Abiotic and biotic factors influencing allelopathic effects on phytoplankton

When released into the aquatic environment allelopathic substances face physical, biochemical and most likely microbial alterations as known for other dissolved organic matter (Scully et al. 2004). The reactivity of phenolic compounds was shown to be affected by solar radiation, temperature and physical and chemical properties of the water, such as pH and oxidation (Appel 1993). Thus phenolic allelochemicals are supposed to be readily degraded or transformed in the aqueous environment with possible consequences for their activity on the target organisms.

1.3.1 Abiotic factors

1.3.1.1 Influence of solar radiation on allelopathic compounds

In the upper surface layers of the water column allelochemicals are exposed to solar radiation. Solar radiation, oxygen and changing redox conditions might influence the stability of allelochemicals (Gross 2003a). Phenolic substances can be easily converted, transformed or degraded by light and oxygen in the aqueous environment (Appel 1993) and thereby change its properties. Photolytic degradation processes were observed for dissolved organic carbon breaking up the high molecular weight substances into easy accessible low molecular weight substanes (Lindell et al. 1995; Wetzel et al. 1995). Enhanced microbial availability of allelochemicals will reduce allelopathic effects on the phytoplankton. In contrast, photolysis can also have negative effects on bacterial bioavailability of dissolved organic matter (DOM), dependent on the origin and specific properties of DOM (Benner und Biddanda 1998; Tranvik und Bertilsson 2001) and manifest possible inhibitive effects. Negative effects on the availability of polyphenols or co-substrates by solar radiation could arise from interrupted degradation processes by polymerization of polyphenols or the formation of recalcitrant degradation products such as high molecular weight substances that are more resistant to further microbial decomposition (Tranvik und Kokalj 1998).

Other dissolved organic compounds that are present in natural waters might also interfere with photolytic and bacterial conversion processes of allelochemicals e.g. labile proteins and humic substances (HS). HS can further be affected by solar radiation with ambivalent consequences for DOM degradation processes and activities of primary producers (Steinberg 2006). Deeper understanding of combined effects of photolytic transformation and bacterial degradation

processes will help to identify allelopathic effects in freshwater systems under changing environmental conditions.

1.3.1.2 Influence of temperature on phytoplankton response

Temperature influences enzyme dependent metabolic processes and thereby might also affect the susceptibility of phytoplankton to allelopathic substances responsible for the observed species specific differences in growth reaction on allelochemicals (Gross et al. 1996; Erhard 2006; Nakai et al. 1999). Bacterioplankton growth and microbial decomposition are optimized at (distinct) optimal growth temperature (Simon und Wunsch 1998). Thus bacteria with degrading activity of the allelochemical or prevailing negative effects on the phytoplankton can be differently proliferate at distinct temperatures. This in turn may influence the sensitivity to allelochemicals or the resistance to allelochemicals. Consequently mutualistic interaction between bacteria and phytoplankton will be changed with temperature. Until now it is not clear which influence temperature has on the sensitivity of phytoplankton species to allelochemicals and which role bacteria play. Depending on how temperature affects the bacteria - phytoplankton interaction the allelopathic effect can be increased or diminished.

1.3.2 Biotic factors

Biotic influences on phytoplankton growth response to allelochemicals can be inter- and intraspecific competition, bacteria or predation pressure or virus infection. In the present work, I focus on bacterial mediation of allelopathic effects, because bacteria interfere with almost all environmental factors *in situ* and complicate the interpretation of allelopathic effects.

1.3.2.1 Bacteria-algae interaction

In the aquatic environment bacteria occur in the surrounding water, attached to the target phytoplankton species and in biofilms in the adjacency of the exuding submerged macrophytes. These bacteria may alter the properties of allelopathic substances and thereby change the allelopathic effect on phytoplankton. Available studies on the effects of allelochemicals on phytoplankton have either used axenic algal cultures or xenic conditions with an undefined bacterial community composition (Gross 2003a). Both approaches impede an assessment of the potential effect of bacteria, which may transform allelochemicals bio-chemically (Smith et al.

2005) or build mechanical barriers such as extracellular polysaccharides (Decho 1990). Bacteria - phytoplankton interaction range from comensalistic, symbiotic to antagonistic e. g parasitic interactions and influence the physiology and community structure of the phytoplankton (Cole 1982; Boyd et al. 1999; Grossart 1999; Kent et al. 2007).

Bacteria can modify the microenvironment of algae (Cole 1982), the so called phycosphere, a contact zone of the phytoplankton with the environment, where exudation products and the mucus of some phytoplankton serve as habitat for specialized bacteria (Fig. 4). Allelochemicals that enter this contact zone have to pass a biofilm composed of exudation products and bacteria until they reach the algal cell surface. Entrance of allelopathic compounds into target organisms depends on specific the physiology of the target organism, e.g. resistance mechanisms and barriers such as cell walls or membranes. Differences in the algal cell wall composition have been proposed as one possible explanation for the variable toxicity of ionic liquids to *Chlamydomonas reinhardtii* (Sena et al. 2010). Cell wall structures, membrane properties, physiological state (cell wall properties differ with developmental stages), alternative transport systems and protective substances (e.g. cell surface associated enzymes or mucus of polysaccharides) might be important for the resistance of target organisms and can explain species specific differences to allelochemicals. Glycoprotein cell walls (Archaea) differ substantially in cell walls composition to green algae, diatoms and blue green algae whose cell walls primarily compose of cellulose, silica or peptidoglycan, respectively (Latala et al. 2005). Membrane disruption or changes of the membrane potential by allelochemicals specifically may thus affect phytoplankton or bacteria. Until now it is not clear if the released allelopathic compounds directly attack phytoplankton species or rather affect bacteria and thereby change their influence on phytoplankton.

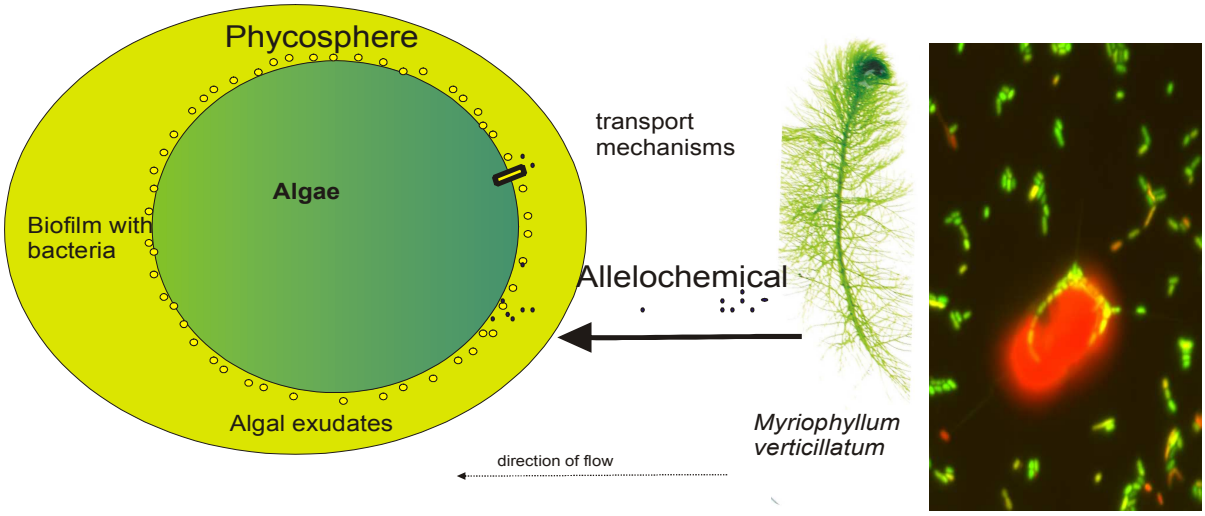


Fig. 4: On the left: Scheme of the phycosphere of the algae surrounded by bacteria (yellow dots) living on algal exudates and interacting with the allelochemical (black dots). On the right: A fluorescence microscopic picture of bacteria in algal culture of the green alga *Desmodesmus armatus* stained by Viability Kit® of Invitrogen. The red fluorescence of the chlorophyll indicates the algae surrounded by a circle of associated bacteria (100000 × magnification).

Bacteria potentially can influence the susceptibility of phytoplankton to allelopathic substances in both directions: negatively by lytic, parasitic or competing bacteria for limited nutrients or positively diminishing the allelopathic effect by degradation or aggregation or support algal growth by additional substrate or micronutrients such as vitamins through heterotrophic mineralization. On the one hand, microorganisms can overcome plant defenses based on tannins (Smith et al. 2005). Changes in bacterial community composition were reported for the upper layer of alpine soils towards a special bacterial community that resists high concentrations of tannin acid (Baptist et al. 2008). Reports of polyphenol degrading bacteria in aquatic ecosystems (Ajay Kumar 1999; Erhan 2002) and different types of phenol catabolism in continuously polluted waters with phenolic compounds (Heinaru 2000) show a high potential of bacteria to detoxify polyphenols enzymatically by biodegradation, via oxidation or to form complexes or polymers. On the other hand, membrane disruption by allelochemicals opens up an entry for the attack of lytic bacteria and virus and can accelerate harm of phytoplankton by existing stressors. Furthermore, toxic by-products of bacteria or more harmful degradation products of the allelochemical can increase allelopathic growth inhibiting effects. Resistant phytoplankton species may benefit from allelopathic effects in the presence of specialized bacteria that can metabolize allelochemicals or use them as co-substrates in nutrient limited habitats.

1.4 Methodological transferability

Differences in detecting allelopathic activity on target organisms may depend on the applied methodological approach. Existing studies on allelopathic substances released by macrophytes mainly focus on effects of cell free exudates, plant extracts or single allelopathic compounds on phytoplankton growth (Mulderij 2007b; Gross 2003a; Gross et al. 2007). *In vitro* experiments are the method of choice to disentangle allelopathic from interference with other processes in the environment but reflect not all aspects of natural performance. Consequently a combination of allelopathic approaches of different complexity was proposed by Gross and colleagues (Gross et al. 2007) to ascertain ecological relevance. Most of the sensitivity tests for allelopathic activity were developed for terrestrial ecosystems and are also used in aquatic systems, e.g. the lettuce seedling bioassay (Elakovich und Yang 1996). However, these tests do not always mirror the complex interaction in the aquatic environment. From an ecological point of view donor and target organisms should belong to the same ecosystem. Detection of allelopathic effects, however, might be difficult due to co-evolution that might lead to development of natural resistance to these compounds as was argued by Reigosa and colleagues (1999). Until now there exists no study that compares the result of different approaches to detect allelopathic activity. This hinders transferability of the results obtained by different approaches and impedes conclusion on community level. The choice of methodology and the chosen target organisms as well as the measured physiological parameters can result in different detectable allelopathic effect and complicate the transferability of results among different approaches.

1.5 Aim of the study

Although in the last decades increasing evidence was given that allelopathy is a structuring element in biotic community composition the knowledge of allelopathic mechanisms in aquatic ecosystems is still fragmentary (Ervin und Wetzel 2003; Gross et al. 2007; Hilt und Gross 2008). An evaluation of the ecological impact of allelopathy requires more detailed studies of aquatic allelopathic interactions in an ecological context. For example, Ervin and Wetzel (Ervin und Wetzel 2003) proposed more ecologically meaningful investigations considering: biotic interaction, chemistry interaction with the environment and unexplained causes of observed allelopathic pattern and potential interactions to occur in the field.

In six articles that are published or soon will be published in international peer reviewed journals (see Publications and presentations) this work addresses the hypothesis 1) that the temporal dynamic of allelochemicals in the plant (*Myriopyllum verticillatum*) is related to growth response of phytoplankton and depend on the nutrient status of the plant, 2) that solar radiation and bacteria influence the allelochemical and its effect on phytoplankton and 3) that abiotic (temperature) and biotic factors such as the presence and the composition of the bacterial community influence algal response to the allelochemical.

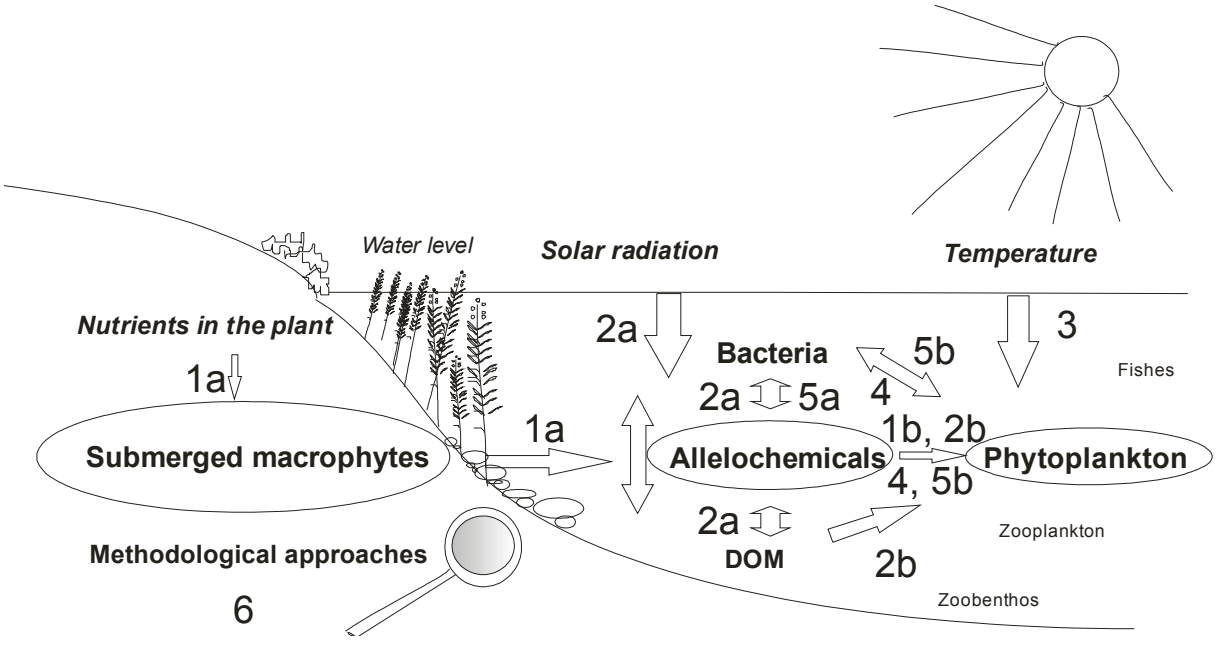


Fig. 5: Selected abiotic (cursive) and biotic factors influencing allelopathic substances and its effects on phytoplankton. Factors investigated in this study are presented in bold letters and addressed interactions indicated with arrows. The Arabic numerals 1-6 indicate the investigated research question.

In order to test the three hypotheses the following **research questions** were addressed in experiments of different complexity and methodolocial approaches from single factor investigation in the lab to multi-method approaches of combined factors to *in situ* experiments (Fig. 5):

1a) *Is there a seasonal and interannual pattern in concentration of polyphenolic allelochemicals in the tissue of Myriophyllum verticillatum and if yes, can it be explained by the nutrient content of the plant?*

1b) *Are changes of the concentration polyphenolic allelochemicals in the tissue of M. verticillatum reflected by algal response in sensitivity tests?*

To answer research question 1a) and 2b) a four year study on *in situ* plant material of *Myriophyllum verticillatum* was performed to investigate seasonal and interannual variations in the total polyphenolic content (TPC) of plant tissue related to nutrient availability. The allelopathic activity of extracted polyphenols was tested in bioassays on *Anabaena variabilis* and the main allelopathic substances were analyzed (Bauer, N; Blaschke, U; Beutler, E; Jenett-Siems, K; Siems, K; Gross, M; Hilt, S, 2009 published in Aquatic Botany). The four year study on the dynamic of total phenolic content of *M. verticillatum* is complemented with parallel measurements for one year by the diplome student Ulrike Blaschke (2006) who established methodology of photometrical TPC determination at the IGB and another year by the diplome student Eike Beutler (2007) supervised by me and PD Dr. S Hilt. Additionally, the data of 2004 generated by PD Dr. S. Hilt (Hilt et al. 2006) were used for this study. Bioassay directed identification of allelopathic activity, fractionation of the main allelopathically active compounds and the analysis of crude extracts by HPLC were done by N. Bauer in cooperation with PD Dr. E.M. Gross at the University of Constance to assure consistence in methodological approaches. The identification of candidates for allelopathic main compounds was conducted with the help of Dr. K Jenett Siems of the FU Berlin and Dr. K. Siems of the Analyticon Discovery GmbH. I wrote this manuscript in cooperation with PD Dr. E. M. Gross and PD Dr. S. Hilt.

2) *Can solar radiation and bacteria influence the diagenesis of polyphenolic allelochemicals in lake water and if yes, does it affect phytoplankton response on allelochemicals?*

To investigate the influence of solar radiation and bacteria on the model substance tannic acid (TA), a known allelochemical from *M. verticillatum* in lake water, changes of the diagenesis of the allelochemical exposed to solar radiation with and without bacteria and subsequent

growth effects are investigated in a full factorial design on the green algae *Desmodesmus armatus* (Bauer, N; Zwirnmann, E.; Grossart, H.-P., Hilt, S., *under revision*, Journal of Phycology). The measurement of the degradation products of TA by size fraction analysis of chromatographic detectable organic carbon based compounds (LC-OCD) was conducted by Dr. E. Zwirnmann at the chemical laboratory of IGB. This study was initiated, designed and performed by myself. I did also data analysis and manuscript writing revised by PD Dr H.P. Grossart, Dr. E. Zwirnmann and PD Dr. S. Hilt.

3) *Does phytoplankton response to allelochemicals change at different temperatures and due to the presence of bacteria?*

The effect of temperature and of bacteria in culture on the species specific sensitivity of phytoplankton towards TA was investigated for three different phytoplankton species at different temperature regimes in the presence or absence of bacteria comparing phytoplankton growth rates of TA treated cultures and parallel controls (Bauer, N; Krienitz, L; Hilt; S *in preparation*). I did the experiments with the help of Max Klomsdorf and Julia Gast during measurements of cell counts and bacterial abundance. Manuscript writing is in preparation.

4) *Do initial algal concentration, bacteria/algae ratio and the presence of specific and unspecific bacteria in culture change allelopathic effects on phytoplankton?*

To investigate the role of bacteria-phytoplankton interaction on the variability of phytoplankton growth response to the allelopathic active substance TA and parallel controls at different algal start concentration, alga/bacteria ratio and with specific bacteria from algal cultures and unspecific bacteria from the nutrient solution were compared (Bauer, N; Grossart, H.-P; Hilt, S, 2008). The study was initiated, performed and the main work on the manuscript was done by myself with the help of PD Dr. H.P. Grossart and revised by PD Dr. D Hilt.

5) *Can changes in the composition of phytoplankton-associated bacteria community and species specific degradation processes of the allelochemicals influence subsequent allelopathic effect?*

The effect of preconditioning of the allelochemical tannic acid (TA) and the effect of natural bacterial communities on TA and subsequent growth rates of the diatom *Stephanodiscus minutulus* were published a study in AME (Bauer, N; Grossart, H.-P., Hilt, S., 2010). I initiated and conducted the study with the assistance of Max Klomsdorff counting the bacteria. The bacteria communities were taken from macrophyte-dominated lake and were pre-adapted with dif-

ferent concentrations of TA. Shifts of bacterial community composition were followed by molecular approaches (PCR, DGGE, and Sequencing) with the help of C, Koppe, K., Hutalle-Schmelzer, C. Dzillas, I. Salka and M. Degebrodt for molecular biological analysis and sequencing. The manuscript was written by myself with the help of PD Dr. H.P. Grossart and revised by PD Dr. S. Hilt.

6) *Are results from different laboratory trials and in situ experiments studying allelopathic effects of submerged macrophytes on phytoplankton comparable?*

A final study comparing different methodological approaches of fluorescence based and particle based parameters is presented that aims to find out if results are transferable between laboratory trials and *in situ* experiments initiated by PD Dr. S Hilt, performed by E. Beutler (i*n situ* and aquaria experiment) and by myself (reagent tube experiments in the laboratory) (Hilt, S; Beutler, E; Bauer, N *under revision*, Journal of Phycology). PD Dr. S. Hilt did the data analysis with the help of myself and wrote the manuscript and revised by myself.

Table 1. List of unspecific groups and identified allelochemical compounds in aquatic macrophytes

Unspecific chemical group	Allelochemical group/ compound	Macrophyte extracted from	Test organism	Reference
	hydrolysable tannins	*Myriophyllum brasiliensis*	*Microcystis aeruginosa, Anabaena flos-aquae*	**(Saito 1989)**
	hydrolysable phenolic acids	*Zostera marina*	*Epiphytic diatoms (e.g. Navicula, Gomphonema, Diploneis)*	**(Harrison und Durance 1985)**
	lipophilic buthylmethylether extract	*Chara aspera*	*Anabaena cylindrical*	**(Berger und Schagerl 2003)**
	glycerids with linoleic acid	*Eichornia crassipes*	*Chlamydomonas reinhartii*	**(He und Ye 1999)**
	phenalene metabolites	*Eichornia crassipes*	*Porphyridium aeruginosum*	**(Della Greca et al. 1992)**
	methanolic extracts (of 0,98% alkaloids, 4,35% phenolic compounds, 1,53% terpenoids) and thin layer chromatographic fractions	*Eichornia crassipes*	*Microalga (Dictyochloropsis splendid, Nostoc pisciale, Clorella vulgaris, Spirulina platensis), bacteria and funghi*	**(Shanab et al. 2010)**
Substance class	**Specific compounds**			
Phenolic compounds	**gallic acid[1,2,4]** **pyrogallic acids[1],** **(+)-catechin[1],** **ellagic acid[1,2,4],**	*Myriophyllum spicatum*	[1]*Microcystis aeruginosa,* [2]*Anacystis nidulans, Chlamy-*	[1]**(Nakai et al. 1999; Nakai et al. 2000; Nakai et al. 2001),** [2]**(Planas et al.**

	tannic acid2, **3,5-dimethoxy-4-hydroxycinnamic acid2,** **protocatechuic acid2,** **shikimic acid2,** **caffeic acid2,** **cinnamic acid2,** **coumaric acid2,** **ferrulic acid2,** **gentisic acid2,** **pyrogallol2,** **quinic acid2,** **sinapic acid2,** **syringic acid2** **galloylglucosides3,** **galloyl ester3,** **derivates of ellagic acid, 3** **tellimagrandin II4**		*domonas globosa, Scenedesmus quadricauda, Selenastrum capricornutum, Euglena gracilis* [3]*Anabaena variabilis, Scenedesmus, Nannochloris, Nitzschia palea, Gomphonema parvulum* [4]*Anabaena sp.PCC7120; Synechococcus sp., Trichormus var., Nanochloris spec., Scenedesmus falcatus, Stigeoclonium tenue*	**1981)** **[3](Gross und Sütfeld 1994)** **[4](Gross et al. 1996)**
	1'0-caffeoyl-6'-0-galloyl-ß-D-glucopyranose, **1'-0-coumaroyl-6'-0-galloyl-ß-D-**	*Myriophyllum verticillatum*	*Synechococcus leopoliensis*	**(Aliotta et al. 1992)**

	glucopyranose, **1'-0-sinapoyl-6'-0-galloyl-ß-D-glucopyranose**			
	1,2-dimethoxy-4-(E-3'methyloxiranyl) benzene **1,2,4-trimethoxy-5-(1'Z-propenyl)benzene,** **1,2,4-trimethoxy-5-(E-3'methyloxyranyl)benzene**	*Acorus gramineus*	*Ankistrodesmus braunii, Chlorella emersonii, Muriella aurantiaca, Stichococcus bacillaris, Euglena gracilis, Pseudococcomixa simplex, Scenedesmus quadricauda, Chlororcoccum hypnosporum, Coccomixa elongata, Chlamydomonas sphagnophila, Chlorella vulgaris, Nostoc commune, Synechococcus leopoliensis, Anabaena flos-aquae*	**(Della Greca et al. 1989)**
	cinnamic acid **dihydrocinnamic acid derivatives,** **C13 nor-isoprenoids,** **1-benzoyl-glycerol-2-alpha-L-arabinopyranoside**	*Schoenoplectus lacustris*	*Selenastrum capricornutum*	**(D'Abrosca et al. 2006)**
	β-sitosterol, **(20S) 24-methylenlophenol,** **stigmast-4-ene-3,6-dione,**	*Thypha latifolium*	*Nostoc commune, Synechococcus leopoliensis, Anabaena flos-aquae, Scytonema hofmanni, Porphyridium aerugineum, Navicula pelliculosa, Chlorella emersonii, Muriella*	**(Aliotta et al. 1990)**

			aurantiaca, Stichococcus bacillaris, Euglena gracilis, Coccomyxa elongata, Chlamydomonas sphagnophila, Chlorella vulgaris, Closterium acerosum, Selenastrum capricornutum, Phormidium autumnale, Porphyriosiphon, notarisii, Aulosira	
	3-(4-hydroxy-3-methoxy)-phenyl 1,2-propandiol, **1-(4-hydroxy-3-methoxy)-phenyl-2-[4-(2,3-dihydroxypropyl)-2-methoxy]-phenoxy-1,3-propandiol**	*Zantedeschia aethiopica*	*Selenastrum capricornutum*	**(Della Greca et al. 1998)**
Unsaturated fatty acids	**2-ethyl-2-methylacetoacetat**	*Phragmites communis*	*Chlorella pyrenoidosa, Ch. vulgaris, Microcystis aeruginosa, Selenastrum capricornutum*	**(Li und Hu 2005)**
	nonanoic cis-6-octadeceanoic acid, **cis-9-octadecanoic acid**	*Myriophyllum spicatum*	*Microcystis aeruginosa*	**(Nakai 2005)**
	α-linolenic acid and its 13-hydroyderivate **linoleic acid and its 9 and 12-hydroxyderivative**	*Zantedeschia aethiopica*	*Selenastrum capriconutum,*	**(Della Greca et al. 1998)**
	linoleic acid,	*Pistia stratoides*	*Plectonema boryanum, Synechococcus leopoliensis, Ana-*	**(Aliotta et al.**

	γ-linoleic acid, **(12R,9Z,13E,15Z)-12-hydroxy-9,13,15-octadecatrienoic acid,** **α- asorone,** **24S-ethyl-4,22-cholestadiene-3,6-dione**		*baena flos-aque, Lyngbya kuetzingii, Phormidium autumnale, Porphyriosiphon notarisii, Aulosira terrestre, Scytonem hofmanni, Porphyridium aerugineum, Navicula pelliculosa, Navicula minima, Ankistrodesmus braunii, Muriella aurantiaca, Stichococcus bacillaris, Scenedesmus quadricauda,Coccomyxe elongata, Closterium acerosum, Selenastrum capricornutum, Chlorella saccharophila*	**1991)**
	dithiolane, **trithiane**	*Chara globularis* *Chara spec.*	*Nitzschia palea,* *natural phytoplankton community*	**(Wium-Andersen et al. 1982)**
Aromatic compounds	**N-Phenyl-2-napthylamine** **N-Phenyl-1-napthylamine,**	*Eichhornia crassipes*	*Chlamydomonas reinhartii*	**(He und Ye 1999)**
Flavonoids	**7-O-diglucuronide of the flavones luteolin,** **apigenin and** **chrysoeriol**	*Elodea canadensis,* *E. nuttallii*	*Anabaena sp., Anabaena variabilis P9, Synechococcus elongatus, Pseudanabaena cf. Catenata, Synechococcus sp., Synechococcus nidulans, Scenedesmus brevispina, Chlorella cf. vulgaris*	**(Erhard 2006)**
	dihydroflavonol,	*Schoenoplectus lacustris*	*Selenastrum capricornutum*	**(D'Abrosca et al.**

	chalcones **(-)-catechin**			**2006)**
Derivates of plant pigments	**2-Ethyl-3-methylmaleimide,** **dihydroactinidiolide** **4-oxo-β-Ionone,** **3-hydroxy-5,6-epoxy-β-ionone,** **loliolide,** **6-hydroxy-3-oxo-α-ionone**	*Vallisneria spiralis*	*Microcystis aeruginosa*	**(Xian et al. 2006)**
Acetylenic compound	**falcarindiol 1 and 2**	*Berula erecta*	*Nitzschia palea*	**(Wium-Andersen 1987)**
Terpene	**four ent-labdane diterpene glycosides,** **two unknown furano-ent labdanes**	*Potamogeton pectinatus*	*Raphidocelis subcapitata*	**(Waridel et al. 2003)**
	two ent-labdane diglycoside	*Potamogeton lucens*	*Raphidocelis subcapitata*	**(Waridel et al. 2004)**
	6 furano-ent-labdanes, **potamogetonin**	*Potamogeton natans*	*Raphidocelis subcapitata*	**(Della Greca et al. 2001)**

	20 ent-labdane diterpenes, for example **15,16-epoxy-12(S)-hydroxy-8(17),** **13(16),14-ent-labdatiren-20,19-olide**	*Potamogeton natans,* *Ruppia maritima*	*Selenastrum capricornutum and consumers (rotifers, crustaceae)*	**(Cangiano et al. 2002)**
	seven ent-labdane diterpenes,	*Ruppia maritima*	*Selenastrum capricornutum*	**(Della Greca et al. 2000)**
Sulphur compounds	**nupharolutine,** **6,6'-dihydroxythiobinupharidine**	*Nuphar lutea*	*phytotoxic activity on lettuce*	**(Elakovich und Yang 1996)**
	elemental sulphur	*Chara globularis* *Chara spec* *Ceratophyluim demersum*	*Nitzschia palea* *phytoplankton and epiphytes*	**(Wium-Andersen et al. 1982; Wium-Andersen et al. 1983)**
	4-methylthio-1,2-dithiolane, **5-methylthio-1,2,3-trithiane**	*Chara globularis*	*Nitzschia palea*	**(Anthoni et al. 1980)**

2 MATERIALS AND METHODS

2.1 Overview of experimental setup

To investigate the different influences explaining variability of allelopathic effects a series of six experiments was performed to answer the six research questions (Fig. 5 and 6) and investigate the temporal variability of allelopathically active compounds of *M. verticillatum* and factors (abiotic, biotic and methodological) influencing the allelochemical and the phytoplankton response to allelochemicals.

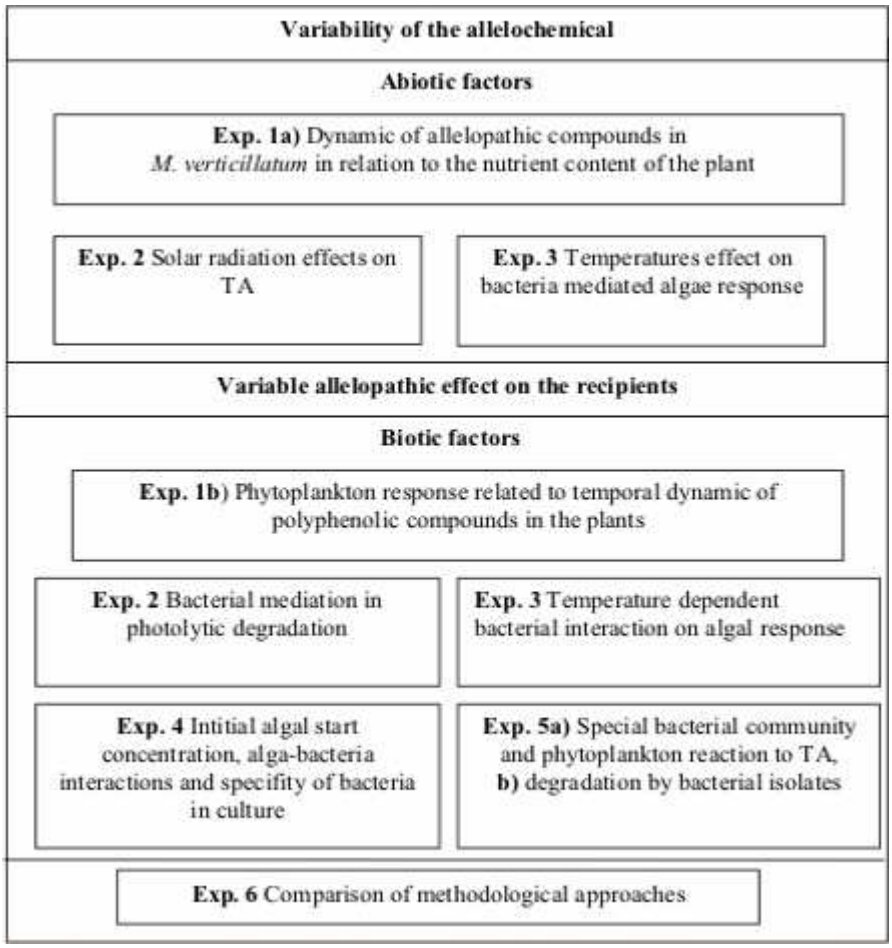


Fig. 6: Overview on experiments performed in this work for studying factors influencing variability of phytoplankton response to allelopathic substances.

2.2 Dynamic of total phenolic compounds in *Myriophyllum verticillatum*

Plant material was sampled from a small, shallow lake, Krumme Laake (52°25.000' N, 13°41.150' E; Berlin, Germany, Fig. 7) with a monospecific stand of Myriophyllum verticillatum L. in a four year period (2004-2007). The lake has an area of about 1 ha and its maximal

depth is 4.8 m [Köhler, 02]. The slightly humic lake is mainly fed by rain and groundwater and DOC concentrations range between 15 and 17 mg L-1 [Beutler, 08]. Physicochemical characteristics are listed in Table 2 for the sampling period from 2004-2007 between May and September, indicating a meso- to eutrophic status.

Table 2: Physicochemical parameter of lake Krumme Laake during the vegetation period of M. verticillatum (May-September) 2004-2007 (n.d.: not determined)

	2004	**2005**	**2006**	**2007**
Water temperature (°C)	13.6-22.6	n.d.	15.9-24.5	14.7-24.3
Secchi depth (m)	0.8-1.2	n.d.	0.70-1.45	0.69-1.22
pH	7.3-8.3	n.d.	6.8-8.2	7.1-8.0
Oxygen concentration (mg l^{-1})	5.4-12.5	n.d.	8.4-11.5	7.8-10.6
Total phosphorus concentrations (µg l^{-1})	36-52	30-45 (May)	34-60	36-59
Total nitrogen concentrations (mg l^{-1})	1.2-1.3	1.1-1.4 (May)	0.6-1.1	n.d.

Fig. 7: Probe site of *Myriophyllum verticillatum* L. in lake Krumme Laake, Berlin, Germany.

2.2.1 Donor organism *Myriophyllum verticillatum* L. (Haloragaraceae)

The Whorled milfoil *Myriophyllum verticillatum* L. (Haloragaceae, Fig. 8) is a perennial submerged macrophyte that forms dense monospecific stands in lakes and rivers (Caffrey und Monahan 2006) due to its dual regeneration by fragments and seed dispersal via hydrochory. It can rapid colonize standing or slow flowing waters by turions that persist in the sediment during winter. The genus Myriophyllum is known for high content of allelopathically active polyphenolic compounds (Gross 2003a). A close relative of *M. verticillatum* L. is the Eurasian milfoil *M. spicatum* L., which contains 7 to 25 % tellimagrandin II, its most allelopathic active polyphenolic substance, and up to 30 % polyphenols in dry mass (Gross et al. 1996; Gross 1995). These polyphenols are considered to play a major role in suppressing phytoplankton growth (Hilt et al. 2006; Gross und Sütfeld 1994) and in plant defence against pathogens and both vertebrate and invertebrate herbivores (Choi et al. 2002; Walenciak 2002; Smolders et al. 2000). *M. verticillatum* possess phenylpropanoid glycosides that were shown to effect growth of the cyanobacteria *Synechococcus leopoliensis* (Aliotta et al. 1992). *In situ*

proof of allelopathic inhibition of distinct phytoplankton species in mono stands of *M. verticillatum* could be reported by (Hilt et al. 2006).

Fig. 8: Habitus a) and b) emergent inflorescences of rhe submerged macrophytes *Myriophyllum verticillatum* L. (Photo: http://flora.nhm-wien.ac.at/Seiten-Arten/ *Myriophyllum-verticillatum*.htm)

2.2.2 Plant material preparation

Plant samples, visually checked for integrity, were collected monthly from May to September. Each month 10 shoots of 25 cm length per replicate were harvested at three different locations in the homogenous plant stand. Shoots were dissected into apices (apical meristems with internode length < 5 mm), leaves and stems, shock-frozen in liquid nitrogen, freeze-dried, homogenized and stored dark and dry at room temperature until further processing. Total phosphorus (TP) and total nitrogen (TN) were measured according to Zwirnmann et al. (1999) in water samples taken in parallel to plant sampling.

2.2.3 Concentrations of TPC, carbon, nitrogen and phosphorus

Per plant sample, 20 mg dry weight (DW) was extracted for 2 h at 16° C with 50% aqueous acetone. The extracts were analyzed for total phenolic compounds (TPC) using the Folin-Ciocalteau assay, modified after Gross et al. (1996) using only 40% of the given volume for sample and reagents. To determine the proportion of non-phenolic compounds reacting with the Folin reagent, TPC content of extracts was measured again after treatment with insoluble polyvinyl polypyrrolidone (PVP; P6755, Sigma Inc., St. Louis, MO, USA). 50 µL of a PVP

suspension (0.1 g mL^{-1} PVP in ultrapure water) was added to 50 µL of extract (raw extract dried *in vacuo*, resuspended in 50% aqueous methanol [v/v]) and shaken for 2 h at 14° C. Carbon (C) and nitrogen (N) concentrations were analyzed in a C/N analyzer (Vario EL, Elementar, Hanau, Germany). Phosphorus (P) content was determined after digestion of 5 mg plant DW with 2 mL H_2SO_4 (5 M) and 2 mL H_2O_2 (30%) for 3 h at 120°C (Zwirnmann et al., 1999).

2.2.4 Fractionation of crude extracts

Crude extracts of *M. verticillatum* apices from 2004 were further separated by solid phase extraction (SPE). 2.25 mL of crude extract was diluted with ultrapure water to a final concentration of 2% MeOH and passed over a preconditioned SPE-C18 cartridge (Varian Bond Elut, 12 mL, 2 g sorbens) with a flow rate of about 3 mL min^{-1}. The cartridge was washed with 20 mL of 2% MeOH and then stepwise eluted with 20 mL each of 2, 15, 20, 25, 30, 35, 75 and 100% MeOH. All 8 fractions were evaporated to dryness and resuspended in 50% MeOH to a final concentration of 100 mg DW mL^{-1}. HPLC analysis and TPC measurements were carried out as described for crude extracts.

2.2.5 HPLC-PDA analysis

To compare the seasonal variability of TPC with that of individual phenolic compounds, extracts of apices (three replicates per month) of the vegetation period of 2004 were analysed by reversed-phase high performance liquid chromatography with photodiode array detector (RP-HPLC-PDA). Aliquots of plant extracts (see 2.2) equivalent to 1 mg plant DW were analysed by HPLC-PDA (JASCO, Gross-Umstadt, Germany; system consisting of an AS1555 auto sampler, a 3-line solvent degasser DG 980-50, a low pressure mixer LG 980-02S, a PU980 pump, a column oven operated at 20°C, and an MD910 photodiode array UV detector). We used a Kromasil-100 C18 column (250 x 4 mm, 5 x 4 mm pre-column; KNAUER, Germany) and solvents A: acetic acid (1% [v/v] in water) and B: MeOH (100%) with an elution profile 0-40 min 5-60% B, 40-45 min 60-100% B, 45-65 min 100% B, 65-67 min 100-5% B, 67-75 min 5% B. UV-absorbing compounds were detected at 280 and 254 nm. The areas of detector signals recorded at 280 nm were used as quantitative measures for concentrations of individual compounds in *M. verticillatum* apices.

2.2.6 LCMS analysis

HPLC separation was carried out on a Perkin Elmer PE Series 200 chromatograph employing a Merck Select B C_{18} column (250 x 4 mm) operating at 23°C. The mobile phase consisted of solvent A (5 mM ammoniumformiate buffer with 0.1% formic acid) and solvent B (acetonitrile: methanol 1:1 with 5 mM ammoniumformiate and 0.1% formic acid); initial conditions were 15% B followed by a linear gradient to 100% B over 30 min at a flow rate of 0.9 mL min^{-1}. Compounds were detected using a UV detector (Merck Hitachi) at a wavelength of 250 nm, an evaporative light scattering detector (ELSD Sedex 75, nebulising temperature 35°C), and by electro spray MS (Sciex API 165, Applied Biosystems, with a turbo ion spray source using MassChrom 1.5.1. software, switching mode between positive and negative electro spray, scanning from m/z 100 to 1500).

2.2.7 Bioassay directed identification of allelopathic plant extract and fraction

2.3 Phytoplankton response to allelopathic substances

The inhibitory activity of crude extracts (raw extract dried in vacuo and resuspended in 50% methanol [v/v] in water) of the apices of M. verticillatum was tested against the cyanobacteria Anabaena variabilis P9 (strain ATCC 29413) using an agar diffusion assay (ADA). The test organism was cultivated and the ADA was prepared as described in Gross et al. [Gross, 91]. Three different aliquots of crude methanolic extracts representing 0.5, 1 and 2 mg plant DW were pipetted onto agar plates (cyanobacteria medium solidified with 1% agar; three replicates per month). The lower agar layer was then overlaid with 7.5 mL of a suspension of A. variabilis (final OD at 530 nm of 0.04 AU per 10 mL) in cyanobacteria medium to which 2.5 mL boiling 4% agar were added, yielding a suspension ready to pour over the base agar. Inhibitory activities could be seen by clearing areas around the spots after 7 days (2004, 2005) or 14 days (2006, 2007) of incubation at 28° C and constant illumination (80 µmol photons m-2 s-1). The inhibitory effects were quantified by measuring the diameter and calculating the area of the clearing zones. Due to different incubation times absolute values of clearing areas can only be compared between seasons of each year, not between years. For comparison, all data of clearing areas of a single year were normalized to 100% (maximum clearing area of the respective year). To identify the major allelopathically active compounds, the inhibitory effect

of different fractions of crude extracts of M. verticillatum apices from 2004, separated by SPE, was determined in ADAs with 2 mg plant DW.

2.3.1 Test allelochemical

In experiment 2-5 and in the reagent tube experiment of experiment 6, tannic acid (100 mL of TA stock solution 1mg/mL, final concentration: 0.002 mg/mL for *D. armatus* and 0.001mg/mL for the diatoms) (TA, Fluka 488119) was added as allelopathic test substance after sterile filtration (PES membrane, 0.2 mm, Whitman, USA) to algae cultures (3 replicates each). TA is a purchasable polyphenol and was used as test allelochemical, because it is one of the allelopathic compounds found in *Myriophyllum spicatum and M. verticillatum* (Lutz 2004) and can be applied in defined concentrations and of constant quality. For parallel controls the same volumina of distilled water (100 µL) were used. The TA solution was prepared freshly each day by ultra sonication of purchasable tannic acid (filling code: 403955/1 64400), Fluka, Germany) in sterile filtered *A. dest* and stored in the dark at 4 °C until further processing. TA addition was performed each day of the sensitivity test at a final concentration of 2µg L^{-1} to *D. armatus* and 1µg L^{-1} to the more sensitive diatoms.

2.3.2 Influence of solar radiation and bacteria on TA and natural DOC

2.3.2.1 Lake water exposure to solar radiation

The allelochemical tannic acid (TA) was added to nutrient enriched lake water and exposed to natural solar radiation (in the following named +light) or kept dark (-light) with (+bacteria) and without bacteria (-bacteria) for up to three weeks. The exposed lake water samples with and without TA and its photolytic and microbial degradation products were chemically analyzed (see 2.3.2.2 *chemical analysis)* at the start of the incubation (t_0), after two (t_1) and after three weeks (t_2) and subsequently tested for their effects on algal growth. Water samples originated from a lake rich in DOC (Krumme Laake, Berlin, 52°25.000' N, 13°41.150' E) that is characterized by a monospecific stand of *Myriophyllum verticillatum* L. The whorled milfoil a submerged macrophyte that contains allelopathically active hydrolysable polyphenols up to 12% of its dry weight (Hilt et al. 2006) among others phenolic acids such as TA (Lutz 2004). The lake water was sampled in July 2008 (pH = 6.8), when the polyphenolic content of *M. verticillatum* is assumed to be high and the natural bacterial community is likely to be pre-

conditioned to contain species that can use these substances as substrates. Water samples were transported in autoclaved 1 L Schott Duran bottles and stored cool until further processing. To remove phytoplankton and zooplankton the lake water was filtered through sterile gauze and passed through a pre-rinsed 3 µm nitrocellulose filter (Millipore, United States). These water samples constituted the +bacteria treatment. In addition, we sterile-filtered (Sterivex-GP filter, 0.22 µm, Millipore, USA) the lake water twice to obtain bacteria free samples (–bacteria treatment). Filtered lake water was then mixed (1:1) with MIIIKS culture solution (Körner and Nicklisch 2002, pH = 8.3) to a final pH of 7.5 ± 0.3 and TA was added (+TA treatment) to a final concentration of 50 mg L^{-1}. Of the pure TA substance 36% of dry mass was detected as carbon by the C/N analyzer. That corresponds to approximately 200 times the natural daily DOC release rate of up to 98 mg C m^{-3} d^{-1} in an oligotrophic lake (Demarty and Prairie 2009). This high concentration was used to overcome methodological limitations in differentiating between added TA and the high background concentration of DOC in the lake water and to follow TA degradation over weeks. TA solution was prepared by ultra sonication of commercially available tannic acid in sterile filtered *A. dest*. (Tannic acid: filling code: 403955/1 64400, Fluka, Germany). Lake water samples with sterile filtered *A. dest.* instead of dissolved TA (-TA treatment) were used as controls. The samples, three replicates for each treatment, were filled (300 mL, 2/5 air) into autoclaved inert Teflon® bags (Layflat FEP Bag 13"x8"x0.005", 96% of solar transmittance including UV, Welch Fluorocarbon, Dover, NH, USA). We sampled each bag once at the start, after two and after three weeks. To minimize DOC contamination, all filters and the bags were pre-rinsed with sterilized deionized water. The bags (O_2, H_2, and CO_2 permeability of 11.6, 34.1, and 25.9 $*10^3$ cm^3 * (m^2 24 h atm)$^{-1}$, respectively) were exposed to natural solar radiation for three weeks (8^{th} to 28^{th} of July 2008) and agitated twice a day to prevent oxygen depletion. The uncovered bags were freely exposed on a grid and received the full natural spectrum of solar radiation recorded during the exposure time. Solar radiation, which reached a daily average of 1670 ± 610 µmol photons m^{-2} s^{-1} of PhAR was measured at a nearby weather station over the whole exposure time. During the incubation we recorded 10 rainy days with a daily average of light intensity of 1390 ± 520 µmol photons $m^{-2}s^{-1}$ of PhAR and 8 days with a daily average of light intensity of more than 2000 µmol photons m^{-2} s^{-1} of PhAR (notice that these are above water level measurements). Temperature recorded by a nearby weather station in Lake Müggelsee varied between 19 and 25°C in 0.5 m depth of the lake and air temperature ranged between 11 and 29°C. The spectral composition of the solar radiation was measured by a spectroradiometer (LI.COR LI-1800,

U.S.A., dominant spectral response 300-1.100 nm). The daily maximum UV radiation (recorded UVB 300, to 320 nm) ranged from 26 to 609 µmol photons m^{-2} s^{-1} on a sunny day. Parallel dark treatments were kept in light impermeable boxes under the same environmental conditions.

2.3.2.2 Chemical analysis

Aliquots of 30 mL were taken from each of the bags exposed to solar radiation or kept in the dark at the start, after two weeks and after three weeks to analyze the degradation products. The samples were passed through well rinsed 0.45 µm cellulose nitrate filter, which had been checked for carbon release (Whatman, United Kingdom) and stored at -20° C until further proceeding. Total chromatographically detectable dissolved organic carbon (CDOC) concentrations as well as the different size fractions of DOC were quantified and characterized by liquid chromatography followed by organic carbon detection (LC-OCD, (Huber und Frimmel 1994; Sachse et al. 2001) as described by Fischer and colleagues (2006). The different size fractions were detected by their relative signal responses at defined retention times (RT). High molecular weight substances (HMWS), which appear first, mostly consist of biopolymers e.g. polysaccharides that were detected at RT up to 35 min. Humic like substances (HS) were detected at 35 to 40 min with "building blocks" appearing up to 43 min. At 46-49 min organic acids and low molecular humics (LMWA) appeared and neutral low molecular weight substances started at 50 min. The DOC fractions occurring at RT of 46-50 min are assigned as low molecular weight substances (LMWS). The specific UV absorption (SUVA) of HS is a measure for HS aromaticity reflecting aromatic and unsaturated structures. There is a close correlation between HS aromaticity and molecular weight of HS (Huber et al., in press). The C fractions were calculated using the software FIFFIKUS (DOC-LABOR Dr. Huber, Germany). The concentration of total phenolic compounds (TPC) was measured photometrically at 750 nm after reaction with the Folin-Ciocalteau reagent (Box 1983). We calibrated with TA in the used lake water plus culture solution and measured with a UV-VIS scanning spectrophotometer (UV-2101 PC, Shimadzu, Japan).

2.3.3 Accompanying bacterial community

2.3.3.1 Bacteria sampling

In experiment 3 and 4 bacteria originate from suspension cultures of algal cultures. Initial bacteria/alga ratios in experiment 4 (1.3–1.8 × 106 bacteria mL^{-1} versus 7.8–14.7 mg Chl-*a* m^{-3} for low and high initial algal biomass respectively) were comparable to those found in freshwater lakes (Cole et al. 1988). Suspended bacteria were achieved from the xenic algal culture by filtration through a 3 µm PC Nucleopore membrane filters.

In experiment 2, 5a and 5b bacteria originated from a natural bacterial community, sampled in autoclaved 1 L Schott Duran bottles from a slightly humic lake (see introduction 2.2., Table 2 for physico-chemical properties, Krumme Laake, Berlin, 52°25.000' N, 13°41.150' E). The lake is characterized by a monospecific stand of *Myriophyllum verticillatum* L. (Haloragaceae).

2.3.3.2 Enumeration of bacteria/ check of bacterial presence in algal culture

In experiment 2, 3, 4, 5a, 5b either axenic single species phytoplankton cultures were checked before and after sensitivity tests for bacterial contamination and if contaminated excluded from experiments or the bacterial abundance was determined by LIVE/DEAD Viability® test (Invitrogen). At the start and the end of pre-treatment and sensitivity test, 1 mL of stained sample was filtered through black 0.2 µm Nucleopore membranes to determine bacterial abundance. Bacteria were stained with the LIVE/DEAD Bacterial Viability Kit (Invitrogen) to check for viable bacteria and with 4', 6-diamidino-2-phenylindole (DAPI, 0.2 mg 100 mL^{-1}; (Porter und Feig 1980) for enumeration under an epifluorescence microscope (Axioskop, 130 VA Typ B, Zeiss, Germany) at 1000× magnification. At least 10 microscopic fields were counted (Grossart 2003).

2.3.3.3 Incubation of bacteria with tannic acid (Exp. 5a, phase 1)

For the pre-treatment of the natural bacterial community water samples from lake Krumme Laake were taken in autumn 2007 (pH = 6.8) when degrading bacterial activity is assumed to be high due to a high amount of decaying plant material. The water was filtered through a sterile 30 µm gauze to remove most of the zooplankton and incubated in autoclaved 500 mL

Erlenmeyer flasks mixed (1:1) with a nutrient rich culture medium (MIIIKS) of pH = 8.3, for nutrient composition see (Körner und Nicklisch 2002), to a final pH of 7.5 ± 0.3. Tannic acid (TA, Fluka, filling code: 403955/1 64400), a hydrolysable polyphenol, was added at **high** (0.5% TA) and **low** concentrations (0.05% TA), whereas controls contained **no** TA. In the following, these treatments are named **H**, **L** and **C**, respectively (Fig. 9). The end of the pre-treatment (**phase 1**) coincided with the first day of the algal culture under TA influence (**phase 2,** Fig. 9). To adapt bacteria to conditions of the subsequent sensitivity experiment (**phase 2**), cultures were incubated for 7 days under constant rotation at 20 °C in a 12:12 h light:dark cycle irradiated with daylight tubes (PhAR) of approximately 90 µmol photons m^{-2} s^{-1} (Osram, 36W/72-965, 830 Biolux, Munich, Germany). To fully remove natural phytoplankton and to minimize protozoan grazing, the water was filtered through 3 µm Nucleopore membranes (Whatman, United Kingdom). Living bacteria and total bacteria were counted at the start and the end of all incubations with TA (**phase 1**) and growth rates were calculated.

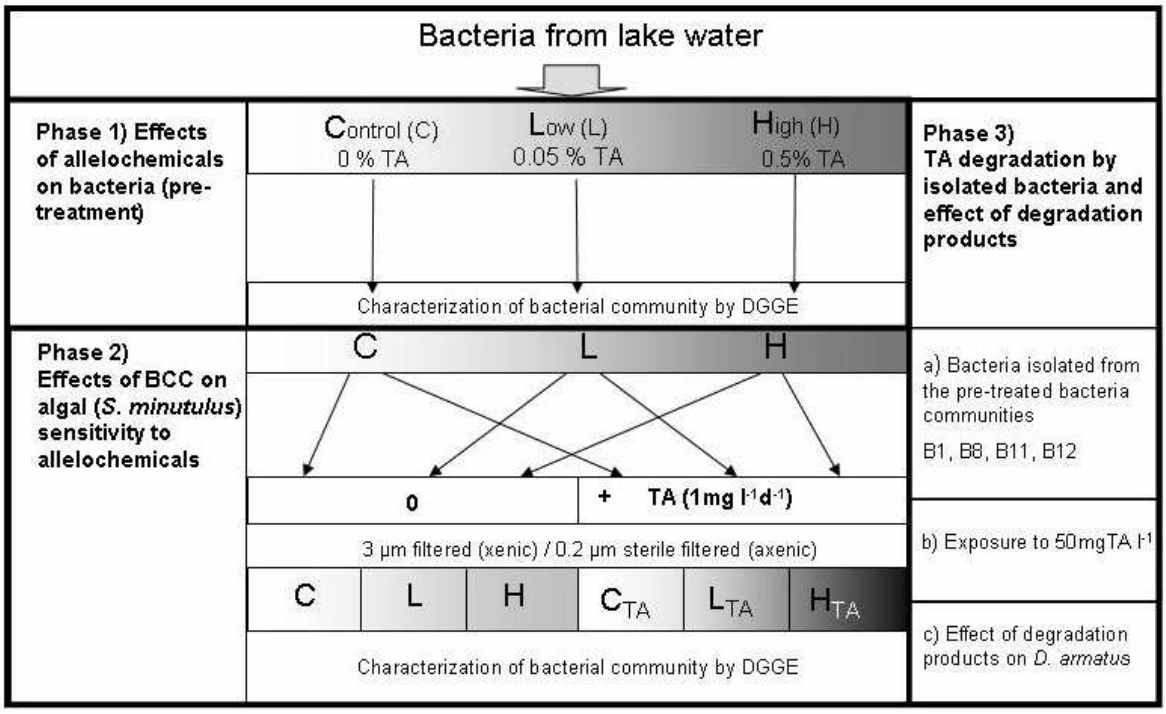


Fig. 9: Experimental design of Exp.5 a, b: 1) **Phase 1**: Pre-adaptation of a bacterial community from lake water exposed to different concentrations of tannic acid (TA) for 7 days (C: control without TA addition, L: low (0.05%) TA concentration, H: high (0.5%) TA concentration). 2) **Phase 2:** Incubation of *Stephanodiscus minutulus* with pre-treated bacteria (xenic) and without bacteria (axenic) but with sterile filtered culture medium of the respective treatments. C, L, H: bacterial community treated with no, low and high TA in the pre-treatment without further TA addition, C_{TA}, L_{TA}, H_{TA}: respective bacterial communities treated daily with 1 mg TA l^{-1}. 3) **Phase 3:** TA degradation by isolated bacteria (a, b) and effects of degradation products (c) on growth and photosynthetic activity of *Desmodesmus armatus*. (Adapted from Bauer et al., 2010)

2.3.3.4 Isolation of polyphenol degrading bacteria (phase 3)

To obtain bacterial isolates for degradation experiments bacteria were isolated from Lake Krumme Laake in October 2007. The isolated bacteria strains were tested for growth on TA as the sole carbon source in medium B (Müller et al. 2007). Isolated bacteria were enriched on DEV-nutrient agar plates (Fluka, filling code: 1124826 54406181, Germany) to enable faster growth of colonies. The plates were cultivated at 16 °C in the dark. Successfull growing cultures were transferred to sterile filtered (0.2 µm Sterivex-Millipore, United States) lake water sampled in June, when allelopathic activity of submerged macrophytes is supposed to be high (Hilt et al. 2006). Cultures were cultivated at 20 °C, and checked weekly for single species culture on DEV-nutrient agar plates. Growth rates of bacteria were determined by enumeration at the start and at the end of incubation following as described for algal growth parameters (2.2.4).

2.3.3.5 Bacterial degradation of TA (phase 3)

For the degradation experiment we selected four bacterial strains (B1, B8, B11, B12) and pre-cultured them in lake water for 4 weeks at 20 °C at the same light conditions to adapt the bacteria to experimental conditions as used in the sensitivity test (**phase 2**) and adjusted to a cell density of 10^6 cells mL^{-1} at the start. They were cultured with TA (50 mg L^{-1} start concentration) and without TA in sterile mixture of lake water (1/3) and MIIIKS (2/3), (pH = 7.5 ± 0.3) for 7 days. After degradation time of a week bacterial cultures were passed through a pre-rinsed and pre-combusted 0.45 µm cellulose acetate membrane (Whatman, United Kingdom) and the remaining TA in solution was detected after reaction with Folin-Ciocalteau (Box 1983) reagent by a UV-VIS Scanning Spectrophotometer (UV-2101 PC, Shimadzu, Japan) as concentration of total phenolic compounds (TPC).

2.3.4 Molecular biology approach

2.3.4.1 Sample preparation

For characterization of bacterial community in experiment 5 150 mL of each treatment were sequentially filtered onto 5.0 µm (for attached bacteria) and 0.2 µm Nucleopore membranes (Whatman, United Kingdom; for free-living bacteria) and stored at -20 °C until further processing (see 2.3.4.1) after pre-treatment with TA.

2.3.4.2 DGGE-analysis and sequencing of bacteria

To characterize the bacteria community composition and find new emerging bacteria during the pre-treatment with TA and during co-incubation with *Stephanodiscus minutulus* in Exp. 5 we performed a DGGE after DNA extraction and PCR amplification of 16S rRNA gene fragments according to (Allgaier 2006). To compare the different DGGE-profiles of the treatments we performed cluster analyses using the software GELCompare II, version 3.5 (Applied Maths), UPGMA and the DICE correlation index. DNA from single DGGE bands was re-amplified for sequencing using the primers GM5 without GC clamp and 907RM and the PCR protocol of (Grossart 2005). Re-amplified DNA was sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems), the IRDye800-labelled primer pair GM5 and 907RM and an ABI Prism 3100-Avant genetic analyser (Applied Biosystems). Sequences were compared with those of reference organisms by BLAST search (http://www.ncbi.nlm.nih.gov/blast). The taxonomy browser of the NCBI server was used for determination of phylogenetic affiliation. All sequences were submitted to the NCBI gene bank (accession numbers GQ332384 to GQ332398).

2.3.4.3 Phylogenetic analysis

Partial 16S rRNA gene sequences were phylogenetically analysed using the ARB software package (http://arb-home.de). The retrieved sequences were imported into an ARB database of 52,000 reference sequences including the closest related sequences determined by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). To increase the robustness of the phylogenetic calculations and to exclude potential alignment errors, a 50% base frequency filter for Eubacteria was calculated.

2.3.4.4 Identification of TA-utilizing bacteria isolates on the basis of 16S rDNA

Twelve isolated bacteria strains were visually checked using an epifluorescence microscope after DAPI staining for formation of single species cell colonies on DEV-nutrient agar plates. Four different isolates were selected for the degradation experiment after DGGE and their DGGE bands subsequently were sequenced (see 2.3.4.2).

2.3.5 Phytoplankton species as target organisms

The selected target organisms were representatives of phytoplankton species common in shallow lakes in Northern Europe, present as well in the sampled lake Krumme Laake. Thus recipients and donor of allelochemical interaction are of the same ecosystem.

In the algae sensitivity bioassays testing the allelopathic effects of TA of experiment 2, 3, 4 and 5b the chlorophyte *Desmodesmus armatus* (Chodat) E. Hegewald (SAG, 276-4e) (formerly *Scenedesmus armatus*), isolated in Germany, Marburg, was used. In culture it forms colonies of two, four, eight and more cells and is characterized by appendices. Sensitivity tests comparing different methodological approaches with allelochemicals from the submerged macrophyte *Myriophyllum verticillatum* and the model substance TA were conducted with the green algae *Desmodesmus subspicatus* (Chodat) Hegewald et Schmidt (UFZ 86.81) and *Stigeoclonium helveticum* Vischer (SAG 116.80) obtained from algae collections at the Environmental Research Centre Leipzig (UFZ) and the Göttingen Algae Collection (SAG) (Fig:10).

Fig. 10: *Desmodesmus armatus* (Chodat) E. Hegewald, *Desmodesmus subspicatus* (Chodat) Hegewald et Schmidt, *Stigeoclonium helveticum* Vischer (SAG 116.80).

Algal bioassays to test allelopathic active TA at different temperatures (Exp. 3) were conducted with two diatoms, the pelagic diatom *Stephanodiscus minutulus* (Kützing) Cleve & Möller (Bacillariophycea, culture collection of the Humboldt University of Berlin, strain HUB 082) also used in experiment 5 and the benthic *Gomphonema parvulum* (Kützing) Kützing (provided axenically by the Collection of Algae (SAG) of Göttingen, order nr.: SAG 1032-1), a representative of the epiphyton of eutrophic waters (Fig.11). Both test organisms represent common diatoms with different life strategies (pelagic, benthic), different temperature preferences. The centric diatom *S. minutulus* is a typical spring representative of phytoplankton and prefers colder temperatures, whereas the pinnate *G. parvulum* was found to grow at higher temperatures up to 34 °C and grow preferably attached to surfaces at (Wallace

1955). *Stephanodiscus minutulus* (Kütz) Cleve et Möller (Bacillariophyta, culture collection of the Humboldt University of Berlin, strain HUB 082) was isolated from Lake Müggelsee situated close to lake Krumme Laake, Berlin, Germany. This diatom prefers cold temperature and is common in spring in northern Europe and was found in 2007 in Lake Krumme Laake (Beutler 2007).

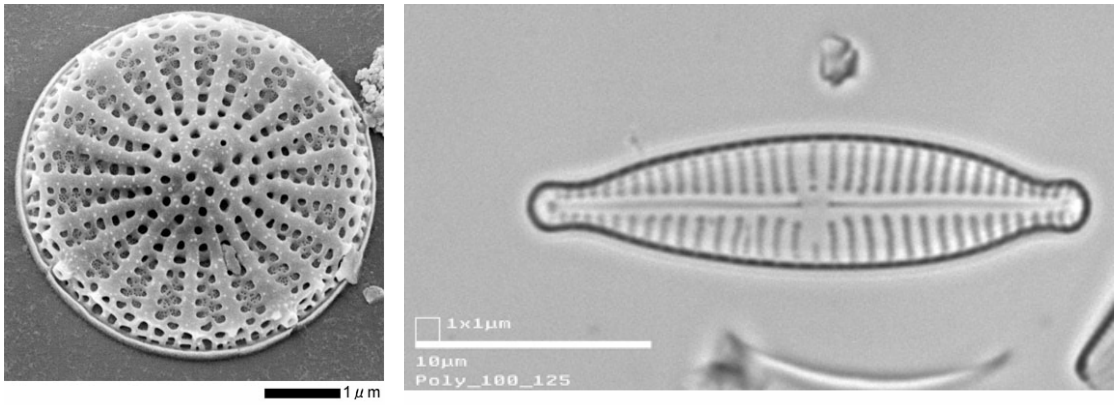


Fig. 11: *Stephanodiscus minutulus* (Kütz) Cleve et Möll., *Gomphonema parvulum* (Kütz) (Photo: www.lbm.go.jp/ohtsuka/atlas/ steminutulus.htm).

For the bioassay directed identification of the main allelopathic active fraction in Exp. 1 the cyanobacteria species Anabaena variabilis Kützing 1843 F ATCC 29413 strain P9 (Fig.12) and Anabaena sp. PCC 7120 were used, which represent common representatives in summer stratified shallow eutrophic lakes.

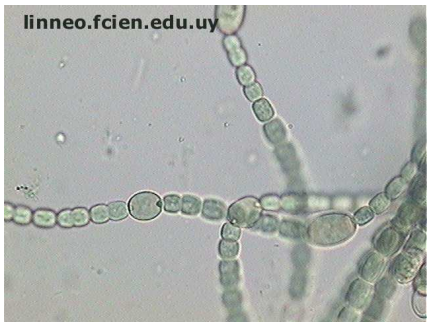


Fig. 12: *Anabaena variabilis* Kützing, (Photo: www.eaubretagne.fr/.../Photos/(categorie)/61875)

2.3.6 Culture solution and culture conditions of tested phytoplankton species

Algal cultures of tested phytoplankton species (see Table 3 for detailed culture conditions for each experiment) were kept in a modified M III nutrient solution [Körner, 02] and were orbitally shaken in 100 mL Erlmeyer flasks at about 60 rpm and at a 12:12 h light: dark cycle. For axenic algal cultures culture medium was enriched with sterile filtered vitamin solution (com-

posed of 1 µg L-1 cobalamin/vitamin B12, 1 µg L-1 biotin, 100 µg L-1 thiamin, final concentration). The culture medium was sterilized by several steps of filtration through a pre-rinsed Sterivex-Units of 0.2 µm pore size (Millipore, U.S.A.) and then passed through a 0.1 µm low protein binding membrane (PVDF Syringe Driven Filter Unit, Millipore, U.S.A.). A scalar irradiance (PhAR) of approximately 90 µmol photons m^{-2} s^{-1} (Osram, 36W/72-965, 830 Biolux, Munich, Germany) was maintained for experiments 1-5. Comparison of methodological approaches (experiment 6) between *in situ*, aquarium and glass tube experiments of the two green algal were performed exposed to natural spectrum of solar radiation for the *in situ* approach and to artificial light that was supplied by fluorescent tubes (Osram Biolux, Munich, Germany) with an emission spectrum similar to daylight for the other approaches. The scalar irradiance (PAR) ranged between 80 to 130 µE•m^{-2}•s^{-1} (see Table 3 for each experiment) at a light period of 12 (experiment 1, 3, 4, 5) to 16 h•d^{-1} experiment (2, 6). Temperature of all experiments were adjusted to 20 ± 1°C and parallel cultivated at 10, 15 °C in the experiment 3.

2.3.7 Algal growth response parameter

Minimal fluorescence (F_0), maximal fluorescence F_m and effective quantum yield F_v/F_m were measured using a XE-PAM (pulse amplitude modulated) fluorometer with white light excitation of a spectrum from UV to near infrared of two xenon flash lamps (Walz, Germany, www.walz.com, for further details see (Körner und Nicklisch 2002). For interpretation of F_0 see (Jakob et al. 2005). Biovolume and algae cell counts as growth parameters independent of chlorophyll fluorescence were determined using a CASY particle counter (CASY®, Modell TTC, measure version 1.5, Schärfe System GmbH, Germany).

Growth rates (µ) were calculated based on F_0 (Körner und Nicklisch 2002) measurements at the start (t_0) and at the end (t_1) of incubation.

$$\mu = \frac{\ln(F_{0t1}) - \ln(F_{0t0})}{\Delta t}$$

µ = growth rate [d^{-1}]

$F_{0\,t0}$ = minimal fluorescence at the beginning of incubation

$F_{0\,t1}$ = minimal fluorescence at the end of incubation

Δ t = time of incubation [d]

This approximation is only appropriate for comparing values of the same algal species under equally maintained culture conditions. Thus measurements of fluorescence parameters were always performed at the same time of the day under identical light conditions. To avoid variance due to different growth phases and self shading low phytoplankton cell numbers (low chl *a* concentrations) were used to ensure exponential algal growth. To avoid background interference with humic substances we used the culture medium with 1/3 lake water for adjustment of the zero offset in experiment 2 and 5.

2.3.8 Algal sensitivity test

The used allelochemical, the donor and target organisms and the different culture conditions of the different bioassays of the experiments 1 to 6 are listed in Table 3.

In experiment 1 an algal diffusions assay (ADA) was performed to identify the most allelopathically active fraction of polyphenolic compounds of M. verticillatum (see 2.2.7).

In experiment 2-6 algal sensitivity tests aqueous culture medium were performed in 100 mL Erlenmeyer flasks or 15 mL reagent tubes (Exp. 2, 5b, 6) under constant gentle rotation in culture solution (MIIIKS see [Körner, 02] for composition of nutrients, pH = 7.5 ± 0.3) in climate chambers in the laboratory .

In experiment 2 previously exposed TA in lake water exposed to solar radiation or darkness with or without bacteria was added to the chlorophyte Desmodesmus armatus to test the different degradation products of TA for growth inhibiting effects on phytoplankton. The algal sensitivity tests were performed before exposure (t0: start), after two (t2) and three weeks (t3) of exposure to solar radiation and bacterial metabolism. We used the axenic green alga Desmodesmus armatus because it showed a broad tolerance towards TA and was sensitive enough to react to TA concentrations as low as 2 µg L-1 (see 2.4). The alga was previously cultured in the culture solution (MIIIKS) and transferred to 1/3 culture solution (MIIIKS) and 2/3 different exposed lake water at start of the sensitivity test. Algae were cultured in 15 mL reagent tubes filled up to 12 mL and gently shaken on a shaken devise that allowed culture of the algae under equal light conditions for 7 days (see Table 3 for further culture conditions). Growth rates were calculated based on fluorescence parameters (see above, 2.3.7). The start concentration of the algae was of 46 µg chl a L-1 (calculated from Chl F0 values and HPLC pigment measurements, see Körner and Nicklisch, 2002).

Table 3. Survey of the applied allelochemical, donor and target organisms and culture conditions of algae sensitivity tests in the different experiments. The variable investigated factor is highlighted using bold letters.

Exp. No	Donor organisms/ Allelochemical	Target organisms	Culture form	Applied bioassay	Bacterial origin	Temperature (C°)	Light (µE•m^{-2}•s^{-1})
1	*Myriophyllum verticillatum*	*Anabaena variabilis P9*	xenic	Agar diffusion assay	unknown	28	80-90
2	TA	*Desmodesmus armatus*	axenic	Suspension cultures in reagent tubes	lake water	20	90
3	TA	*Desmodesmus armatus, Gomphonema parvulum, Stephanodiscus minutulus*	axenic, xenic, axenic, xenic, xenic	Suspension cultures in Erlenmeyer flasks	algal culture	10, 15, 20	
4	TA	*Desmodesmus armatus*	axenic, xenic	Suspension cultures in Erlenmeyer flasks	culture medium	20	90
5	TA	*a) Stephanodiscus minutulus* *b) Desmodesmus armatus*	axenic, with special bacterial communities	Suspension cultures in Erlenmeyer flasks	lake water, pre-adapted to TA	20	90
6	TA	*Desmodesmus subspicatus,* *Stigeoclonium helveticum*	xenic, xenic, xenic	Suspension cultures in reagent tubes, aquaria test, *in situ* dialysis bags	algal culture	20	130

In order to investigate the influence of temperature (**experiment 3)** on algal response to TA the two diatoms (*Stephanodiscus minutulus, Gomphonema parvulum*) and the green algae (*Desmodesmus armatus*) were previously acclimatized for three weeks to the experimental

temperature conditions of 10, 15 and 20°C. The axenic (-bacteria) and xenic (+bacteria) cultures of *Gomphonema parvulum* and *Desmodesmus armatus* were cultured parallel under defined growth conditions (Table 3) to find differences in growth response to TA due to the presence of bacteria. Algal cell counts and growth rates were calculated based on fluorescence parameters (see above 2.3.7) at the start of incubation and after 6 days and the bacteria to alga ratio was calculated.

In **experiment 4**, three different cultures of *D. armatus* were used in the algal suspension test in Erlmeyer flasks: (1) without bacteria; (2) axenic algal culture inoculated with unspecific suspended bacteria adapted to the culture solution MIIKS (unspecific bacteria); and (3) with specific bacteria adapted to the alga co-cultured for several generations with the green alga *D. armatus* cultures (specific bacteria). The experiment was performed parallel with low algal start concentration (7.8 mg Chl-*a*/m^3) and the doubled high algal start concentration (14.7 mg Chl-*a*/m^3) resulting in a lower effective TA concentration per alga cell and a lower initial bacteria/algae ratio. The algal sensitivity tests were performed in Erlenmayer flasks under controlled conditions (Table 3) and growth rate was calculated based on fluorescence and biovolume as described above (2.3.7.).

In **experiment 5a** effects of different, pre-treated bacterial communities on algal sensitivity to allelochemicals were tested on the growth of the axenic diatom *Stephanodiscus minutulus* cultured in 1/3 bacteria inoculum or bacterial filtrates to 2/3 culture solution (MIIIKS, pH = 7.5 ± 0.3). Inoculates of 20 mL bacteria (pre-treated with 0% TA: control (C), 0.05% TA: low TA concentration (L) and 0.5% TA: high TA concentration (H) (Fig. 10), adjusted to 10^6 cells mL^{-1}) were added to 40 mL axenic *S. minutulus* (see above 2.2.6.3, **phase 2**, Fig. 10). The diatom was kept in batch culture with a low start concentration to ensure exponential growth over most of the incubation time. The algae start concentration was adjusted to (F_0) = 5 using a Phyto-PAM fluorometer (Walz, Germany). This corresponds to a chl *a* content of about 25 µg L^{-1} following (Körner und Nicklisch 2002). The axenic and xenic diatoms were cultured in 100 mL Erlenmeyer flasks under constant gentle rotation. Semi-continuous addition of TA (0.001 mg TA mL^{-1} d^{-1}) to *S. minutulus* (C_{TA}, L_{TA}, H_{TA}, phase. 2, Fig. 10) and sterile filtered distilled water as controls (C, L, H, phase. 2, Fig. 10) lasted for 7 days in the presence and absence (axenic filtrates) of different pre-treated bacterial communities. Three replicates were conducted for each treatment.

In **experiment 5b** effects of bacterial degradation products of TA on algae growth rates were tested on axenic cultures of the chlorophyte *Desmodesmus armatus* (Chodat) E. Hegewald (SAG, 276-4e), because of its broader tolerance at high TA concentrations (experiences of former biotests). The culture medium of the bacteria with and without TA was sterile-filtered twice (0.2 µm Sterivex, Millipore) and added to axenic *D. armatus* cultured in reagent tubes. All tubes were filled to 3/4th of the glass tubes and permanently rotated to prevent lack of oxygen. F_0 was measured at the start of incubation with TA degradation products and after 7 days of exposure to the degradation products to calculate growth rates as mentioned above.

For **experiment 6** three different approaches were performed to test for differences in the sensitivity of phytoplankton response to evaluate weather results of a) *in situ* and b) coexistence experiments with *M. verticillatum* in aquaria and c) glass tubes experiments with addition of 25 µg L^{-1} TA are transferable independent from applied methodology. The different approaches were separately performed (a) co-incubation of algae in dialysis membrane tubes in a lake inside and outside a *M. verticillatum* stand, (b) coexistence approach in aquaria with and without shoots of *M. verticillatum* and (c) single additions of tannic acid, an allelopathically active polyphenol present in this macrophytes (Lutz 2004), to the algae cultures. For each method, fluorescence (chl *a*, photosystem II activity)- and particle (cell count, biovolume)-based parameters were compared after 48 hours of incubation.

Table 4. Water quality parameters at the end of the experiments in Lake Krumme Laake, aquaria and glass tubes (control/treatment, means ± standard error,* significantly different at $p<0.05$, t-test). Data for nutrients and pH in aquaria were taken from similar experiments conducted in Hilt & Lombardo (2010) and Hilt et al. (2006), respectively, in tubes only starting values are available. (Hilt S., Beutler E., Bauer N. under revision).

Parameter	**Lake**	**Aquarium**	**Tube**
Water temperature (°C)	18.7 ±0/18.3 ± 0.02*	20 ± 0.1/20 ± 0.1	20 ±0.1/20 ± 0.1
pH	7.0 ± 0.1/7.1 ± 0.1	8.3 ± 0.02/8.6 ± 0.01*	8.3 ± 0.1
Soluble reactive phosphorus concentration (µg L^{-1})	<3	1400 ± 50/1035 ± 40*	1550
Soluble inorganic nitrogen concentrations (mg L^{-1})	0.03 (NH_4-N)	7.5 ± 0.3/4.7 ± 0.3* (NO_3-N)	8.15 (NO_3-N)

a) In situ test with test organisms in dialysis tubes

Both target species were cultivated in a modified MIIIKs nutrient solution, for details see (Körner und Nicklisch 2002), for 48 hours exposed in dialysis membrane tubes (4 replicates) inside and outside a *M. verticillatum* stand in Lake Krumme Laake (water quality Table 4) in early July 2007, when macrophytes filled the entire water column in their stand. The pH was 8.3 in equilibration with air (Table 4). The nutrient solution is very rich in phosphorus allowing polyphosphate accumulation that is assumed to support growth of several algae generations in the absence of external P (John und Flynn 2000). Slow release fertilizer (OSMOCOTE), 3 g per dialysis tube, was added to the dialysis tubes to prevent nutrient competition between algae and plants (Worm et al. 2000). Detailed descriptions of the methods are available in (Hilt et al. 2006) and (Körner und Nicklisch 2002).

b) Incubation test with *M. verticillatum* in aquaria

Cultivation of both green algae was conform to *in situ* test with dialysis bags, however, dialysis bags were exposed in a 4 L aquarium containing M III nutrient solution and 20 shoots (25 cm) of *M. verticillatum* (9 g fresh weight L^{-1}, a density comparable to field situations (Grace und Wetzel 1978) from Lake Krumme Laake (harvested in July 2007, 4 days before the experiments) or plastic plants of similar density (to simulate the shading effect) as controls.

c) Laboratory tests in reagent tubes with TA

In the laboratory approach the two green algae were exposed to a single additions of 250 µL tannic acid (Fluka BioChemika, Steinheim, Germany, 25 $\mu g \cdot mL^{-1}$) or distilled water as a control to 9.75 mL algae cultures (in M III nutrient solution) in glass tubes (4 replicates, placed on a shaking device) for 48 hours.

2.4 Data analysis

In **experiment 1** the TPC, C/N ratio, TN and TP concentrations as well as bioassay data for seasonal and interannual differences were tested using non-parametric Kruskal-Wallis-tests and subsequent multiple comparisons using Mann-Whitney-U tests with p=0.05 divided by the number of all possible comparisons. Mann-Whitney-U tests were also used for detecting the effect of treatment compared to control in bioassays. Since different plant parts (apical tips, leaves and stems) significantly differed in their TPC, C, TN and TP contents (see results), the effect of year and season was tested separately for each plant part. Tests of correlation for

TPC and C/N, TPC and TP and TPC and mean water temperature as well as global radiation were performed using Spearman's rank correlation at $p<0.05$. To compare HPLC peak areas during different seasons and TPC in apical tips of different water depths we used one-way analysis of variance (ANOVA) and subsequent Tukeys posthoc tests (when appropriate) at $p<0.05$. All data analysed by parametric tests were tested for normal distribution and homogeneity of variance.

In **experiment 2** the influence of light and bacteria on total CDOC and single DOC size fractions was analyzed after 2 weeks of incubation by performing multiple analyses of variance (MANOVA) separately for the treatments with and without TA. To further differentiate according to combinations of treatments '+/-light' and '+/- bacteria' the same data set and growth rate of algae cultured in the treated water were analyzed separately for lake water with and without TA by using one-way ANOVA and the subsequent Scheffé post hoc test.

In **experiment 3** differences of algal growth rates and bacterial abundance between temperatures were analyzed separately for algal cultured– and + bacteria with and without TA (+/-TA treatment) by one way analysis of variance ANOVA with subsequent Tukey/Scheffé post hoc test. If differences in growth rate/bacterial abundance were not normally distributed non-parametrically Kruskal Wallis test was applied. For the diatom *G. parvulum* differences to control data had to be LN transformed in the bacteria free treatments and square root transformed in the treatment with bacteria. Differences between control and TA treatment were tested against zero using Mann Whitney test stated as differences if $p < 0.0.5$.

In **experiment 4** statistical comparisons between treatment and control were carried out by Student's t-test at $p < 0.05$, and differences between control and treatment were compared for ax, sb and x using one-way ANOVA and subsequent Tukeys posthoc tests at $p < 0.05$.

In **experiment 5a, b** to compare between bacterial growth according to pre-treatment with different TA concentrations (phase 1); algal growth rates treated with different bacterial inocula/ filtrates of pre-treatments (phase 2); and to test for differences of bacterial degradation of specific bacterial isolates comparing TPC concentrations and among the untreated bacterial filtrates (phase 3) we performed a one-way ANOVA with subsequent Tukey's posthoc test. Differences in growth rate between TA treatment and control were tested with Student's t-test.

In **experiment 6** means of control and *M. verticillatum* treatments at the end of different methodological approaches were compared for each parameter using Student's *t*-test to asses the allelopathic influence of *M. verticillatum* and TA on the algae. To compare methods (lake,

aquarium, tube) a one-way ANOVA and subsequent Tukeys posthoc tests were carried out for both algae species using the percentage differences between control and treatment (*M. verticillatum* or TA) for each parameter (chl *a*, PS II activity, cell counts, biovolume) at the end of the experiment. To compare the sensitivity of the species a two-way ANOVA was carried out using the percentage differences between control and treatment for each parameter with method and species as fixed factors. Data on PS II activity had to be cube transformed to obtain homogeneity of variances.

The statistical package of PAS 17.0 for Windows, SPSS inc. Chicago L, USA was used for all statistical analysis.

3 RESULTS

3.1 Dynamic of total polyphenolic compounds in *Myriophyllum verticillatum*

To investigate the temporal variation of the content of potentially allelopathic substances in the submerged macrophyte *Myriophyllum verticillatum* total phenolic content of plant material was measured harvested monthly from lake Krumme Laake (see 2.2 *Material and Methods* for procedure and physico-chemical parameter of the lake) for the period of 4 years.

3.1.1 Temporal dynamic of TPC and C/N ratio, N and P concentrations

TP and TN concentrations in the water of Lake Krumme Laake during the vegetation period of *M. verticillatum* from May to September 2004-2007 ranged between 30 – 60 µg L^{-1} and 0.6 – 1.4 mg L^{-1}, respectively, and were not significantly different between years. TPC as well as C/N ratio, N and P concentrations significantly differed between apices, leaves and stems of *M. verticillatum* with highest TPC, N and P concentrations in apices (Fig. 13, Table 5).

Table 5: Differences in concentrations of total phenolic compounds (TPC), C/N molar ratios and nitrogen (N) and phosphorus (P) contents between apices, lower leaves and stems of *M. verticillatum* between May and September 2004-2007. Data represent medians, those data sharing the same letter in one row are not significantly different at $p < 0.017$ (Kruskal-Wallis-test, multiple comparisons at $p < 0.05$/number of comparisons (3), N = 180).

	Apices	**Leaves**	**Stems**	**p**
TPC (mg g^{-1} DW)	76 c	48 a	56 b	<0.0001
C/N molar ratio	12.0 a	13.1 b	19.2 c	<0.0001
N (mg g^{-1} DW)	33.7 c	30.4 b	18.9 a	<0.0001
P (mg g^{-1} DW)	3.71 b	2.21 a	2.51 a	<0.0001

The highest (120 mg g^{-1} DW in apices in May 2004) and lowest concentration of TPC (17 mg g^{-1} DW in leaves in May 2005) varied almost by an order of magnitude in four years. Addition of Polyvinylpyrolidon to complex with phenols to the assays revealed on average 13% (range 4-22%) non-phenolic compounds being detected as TPC. Significant interannual differences were recorded for all plant parts for TPC (Table 6), but not for C/N ratio, N and P concentra-

tions (Kruskal-Wallis-test, $p < 0.05$), when data of all seasons were pooled. Concentrations of TPC in apices and leaves were highest in 2004 (Table 6).

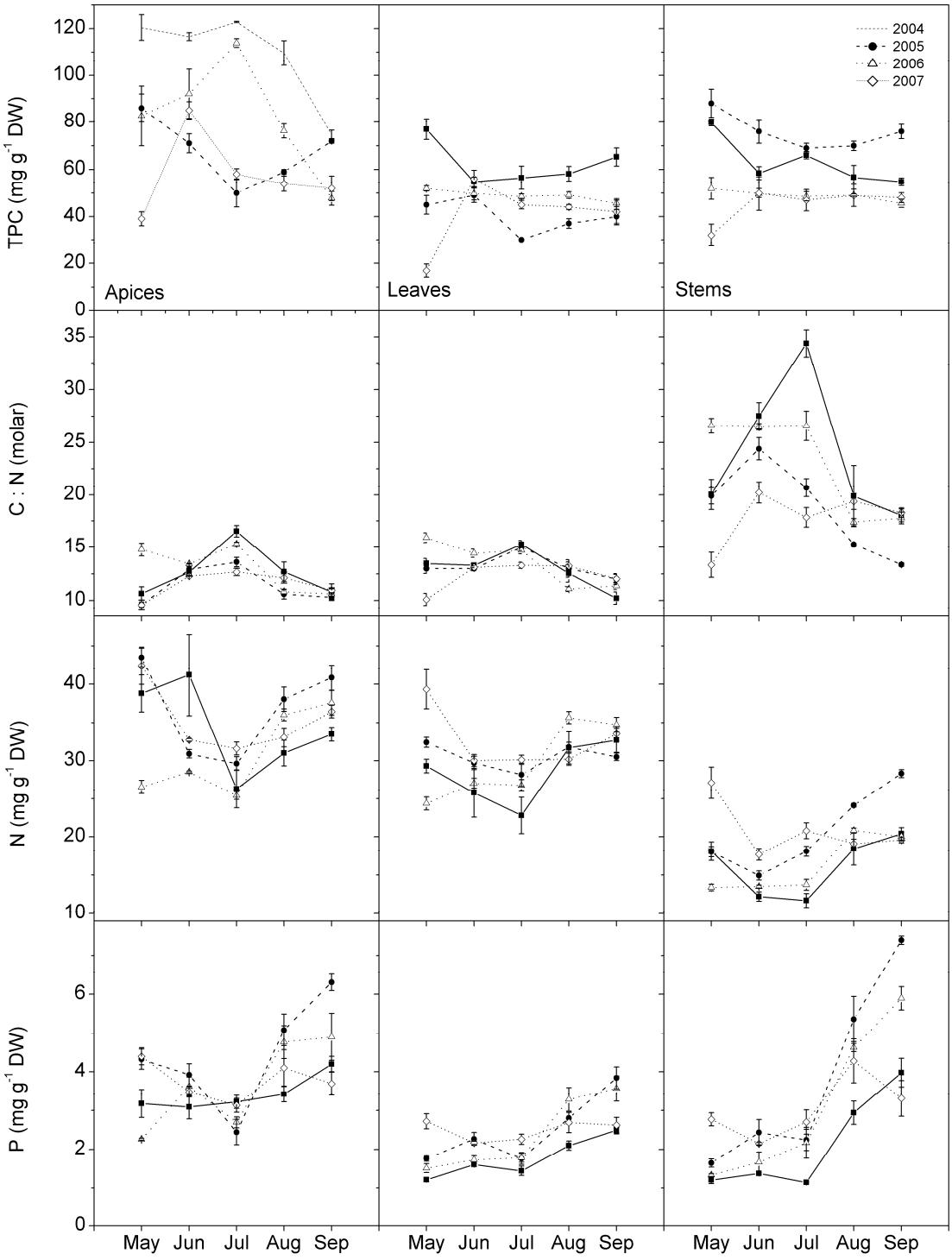


Fig. 13: Seasonal patterns of total phenolic compounds (TPC), C/N molar ratio, total nitrogen (TN) and total phosphorus (TP) content in *Myriophyllum verticillatum* shoots from Lake Krumme Laake (Berlin, Germany) during the growing seasons 2004-2007. Data presented are means ± standard error (n = 3).

Interannual differences in TPC were strongest in May (Fig. 13). Significant monthly differences of the growing season were measured for all plant parts for C/N ratios, N and P concen-

trations, but not for TPC when data of all years were pooled. We found highest C/N ratios and lowest N concentrations in June and July and increased P concentrations in all plant parts towards the end of the growing season (Kruskal-Wallis-test, $p < 0.0001$, multiple comparisons at $p < 0.05$/number of comparisons (10), N = 180). Correlations between TPC and C/N and between TPC and P were mostly weak or not significant (Table 7). Pooling data of all years for the individual plant parts resulted in a weak positive relation between TPC and C/N ratio for apices and leaves (although an inverse relation was found for apices in 2005), and a weak inverse relation between TPC and P concentration (Table 7).

Table 6: Interannual differences in concentrations of total phenolic compounds (TPC) in apices, lower leaves and stems of *M. verticillatum* between May and September 2004-2007. Data represent medians, those sharing the same letter in one line are not significantly different at $p < 0.008$ (Kruskal-Wallis-test, multiple comparisons at $p < 0.05$/number of comparisons (6), N = 180).

		2004	**2005**	**2006**	**2007**	**p**
TPC (mg g^{-1} DW)	**Apices**	116 c	69 ab	91 bc	55 a	<0.0001
	Leaves	60 c	39 a	49 b	44 ab	<0.0001
	Stems	64 b	74 c	47 a	46 a	<0.0001

Table 7: Correlations (Spearman Rank, r: correlation coefficient) between total phenolic compounds (TPC), C/N molar ratios and phosphorus contents (P) in apices, lower leaves and stems of *M. verticillatum* between May and September 2004-2007 (bold: significant at * $p < 0.05$ or ** $p < 0.01$).

		Apices		**Leaves**		**Stems**	
		r	**p**	**r**	**p**	**r**	**p**
TPC-C/N	**2004**	0.519	0.051	-0.147	0.602	0.394	0.146
	2005	-0.592*	0.020	-0.106	0.708	-0.075	0.790
	2006	0.831*	<0.0001	0.364	0.182	0.386	0.156
	2007	0.536*	0.040	0.654*	0.008	0.611*	0.016
	2004-2007	0.429**	0.001	0.265*	0.041	0.227	0.081
TPC-P	**2004**	-0.629*	0.012	-0.247	0.375	-0.686*	0.005
	2005	0.363	0.183	0.104	0.712	-0.201	0.473
	2006	-0.639*	0.010	-0.407	0.132	-0.232	0.405
	2007	-0.296	0.283	-0.654*	0.008	-0.443	0.098
	2004-2007	-0.382**	0.003	-0.357**	0.005	-0.267*	0.040

3.1.2 Seasonal dynamics of individual compounds

We found nine major compounds by HPLC-PDA analysis of extracts of *M. verticillatum* apices of 2004 (Fig. 14). Compound no. 1 with a retention time of 7 min was identified as gallic acid based on its UV spectrum and co-chromatography with the pure compound (Lutz 2004). The other major HPLC-PDA signals refer to several hydrolysable tannins, among them gallo- and ellagitannins and at least one flavonoid glycoside based on preliminary LCMS analyses (Tables 8, 9). Most of the major compounds showed a clear seasonal pattern with highest concentrations present in May and lowest in September (Fig. 14, one-way ANOVA at $p < 0.05$, Tukeys posthoc test).

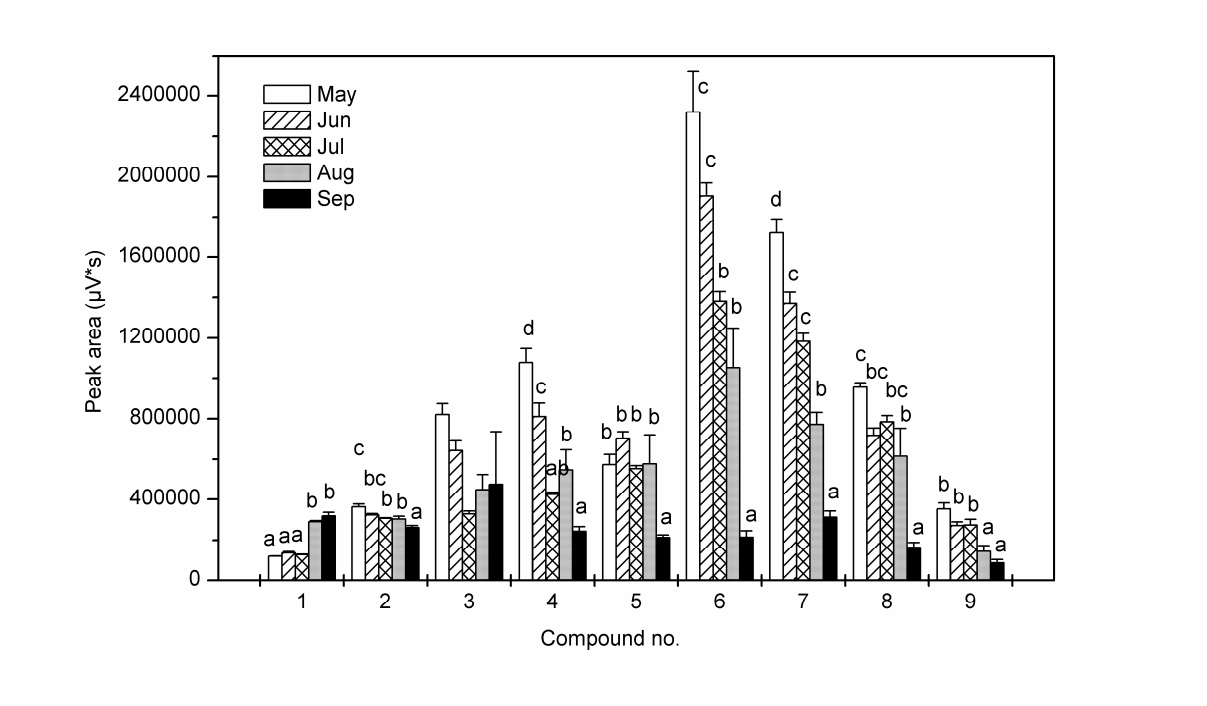


Fig. 14: Dynamics of HPLC-PDA peak areas of 9 main compounds of extracts from apices of *M. verticillatum* 2004 that correspond to 100 mg plant dry weight. Data presented are means ± SE (n = 3). Different letters indicate significant differences between seasons (one-way ANOVA, Tukeys posthoc test at $p < 0.05$).

HPLC-PDA peak areas of six compounds were positively correlated with TPC (Table 9). Gallic acid showed an opposite pattern and peak areas were inversely related to TPC (Fig. 14, Table 9). None of the compounds significantly correlated with the C/N ratio, but six were inversely correlated with P (Table 9). In fact, correlation coefficients of HPLC areas of these individual phenolic compounds with P were similar to those of TPC with P (Table 7, Table 9).

Table 8: Polyphenolic compounds detected in apices of *M. verticillatum* in 2004 (HHDP: hexahydroxydiphenoyl). * No. referring to the nine major compounds and the related number in Fig. 14.

No. *	Retention time in HPLC-PDA (min)	Retention time in LCMS (min)	$[M-H]^-$	Compound
4	**17**	7.03	785	HHDP-di-galloylglucose
5	**20**	7.70	635	Tri-galloylglucose
6	**22**	8.32	785	HHDP-di-galloylglucose
7a	**24**	9.09	785	HHDP-di-galloylglucose
7b	**25**	9.97	937	HHDP-tri-galloylglucose
8	**28**	10.20	937	HHDP-tri-galloylglucose
9	**35**	13.74	447	Quercetin-glycoside

Table 9: Correlations (Spearman Rank) between peak areas of HPLC-PDA signals of 9 main compounds and total phenolic compounds (TPC), C/N molar ratios and phosphorus contents (P) of *M. verticillatum* apices between May and September 2004. (n. i.: not identified, HHDP: hexahydroxydiphenoyl).

No.	Name	TPC		C/N		P	
		r	p	r	p	r	p
1	**Gallic acid**	-0.726**	0.002	0.005	0.985	0.450	0.092
2	**n. i.**	0.616*	0.014	-0.127	0.652	-0.643**	0.010
3	**n. i.**	0.050	0.859	-0.188	0.503	-0.361	0.187
4	**HHDP-di-galloylglucose**	0.486	0.066	-0.252	0.365	-0.646**	0.009
5	**Tri-galloylglucose**	0.649**	0.009	0.331	0.229	-0.657**	0.008
6	**HHDP-di-galloylglucose**	0.660**	0.007	-0.075	0.790	-0.582*	0.023
7	**Mixture of HHDP-di- and -tri-galloylglucose**	0.631*	0.012	-0.123	0.661	-0.636*	0.011
8	**HHDP-tri-galloylglucose**	0.783**	0.001	0.039	0.889	-0.471	0.076
9	**Quercetin-glycoside**	0.712**	0.003	0.027	0.924	-0.625*	0.013

3.1.3 Allelopathic activity of *M. verticillatum* extracts and SPE fractions

Crude extracts of apices of *M. verticillatum* of all years significantly inhibited the growth of *A. variabilis* as compared to MeOH controls in the ADA when applied in equivalents of 2 mg plant DW (Fig. 15, Mann-Whitney-U-tests at $p < 0.05$). The application of 0.5 mg plant DW only resulted in a significant inhibition in 2006 (except for June), and that of 1 mg plant DW in 2005 (all seasons), 2006 (except for June) and 2007 (only June) (Fig. 15). A significant correlation between TPC and the clearing area of crude extracts of *M. verticillatum* apices was found for all years except 2005 (Spearman rank correlation, 2004: $r = 0.692$, $p = 0.004$; 2006: $r = 0.628$, $p = 0.012$; 2007: $r = 0.628$, $p = 0.012$). Significant seasonal differences in the inhibitory activity (of 2 mg DW) were found only in 2005 and 2007, and were weaker in the other two years (Kruskal-Wallis test, 2004: $p = 0.064$; 2005: $p = 0.017$; 2006: $p = 0.094$; 2007: $p = 0.045$).

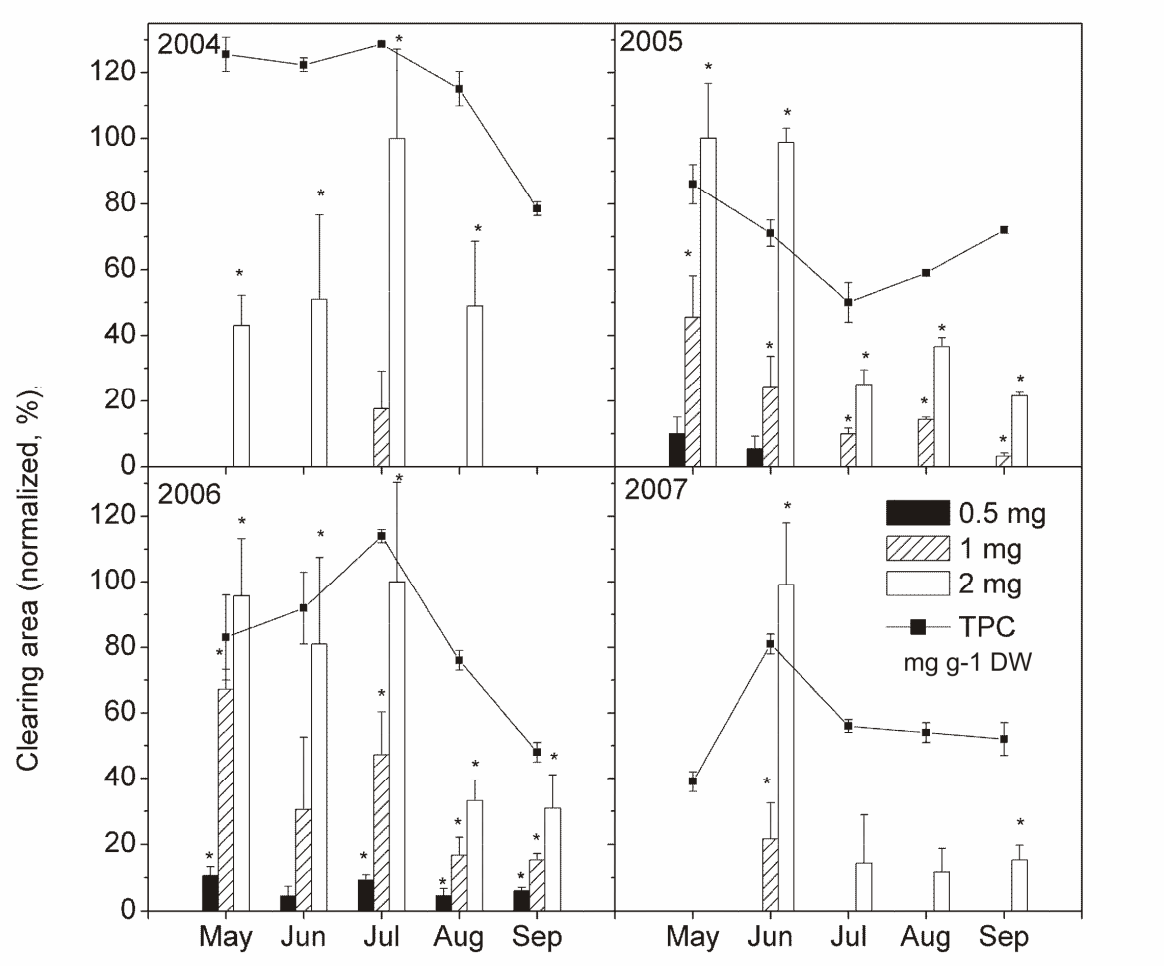


Fig. 15: Extent of the inhibition (normalized clearing areas) of *Anabaena variabilis* P9 in the agar diffusion assay of different amounts of crude extracts of apices of *M. verticillatum* during the growing seasons 2004-2007 plotted together with the seasonal variation of the concentrations of total phenolic compounds (TPC). Data presented are means ± SE (n = 3). * Significantly different from control (Mann Whitney U test at $p < 0.05$).

Significant inhibitory effects of SPE fractions of *M. verticillatum* apices from 2004 on the growth of *A. variabilis* were found in four fractions: 25%, 30%, 35% and 75% MeOH (Fig.16, Mann-Whitney-U-tests at $p < 0.05$). These fractions mainly contained the compounds no. 6 (25%), 6 and 7a/b (30%), 7a/b and 8 (35%) and 9 (75%) (Table 8). A significant correlation between TPC and clearing area was found when pooling fractions 25%, 30% and 35% (Spearman rank correlation, $r = 0.918$, $p < 0.001$).

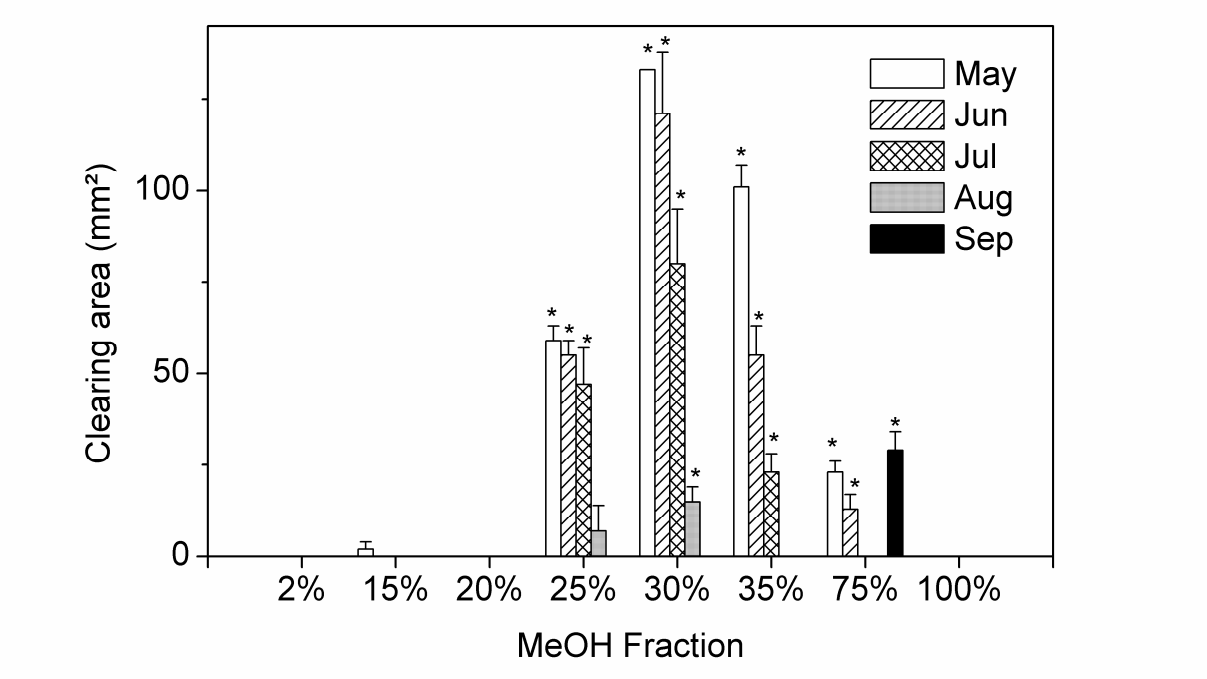


Fig. 16: Seasonal variation during the growing season 2004 of the inhibition of *Anabaena variabilis* P9 in the agar diffusion assay of different chromatographic fractions of crude extracts eluted with different percentage of MeOH by solid phase extraction of apices of *M. verticillatum* corresponding to 2 mg plant dry weight. Data presented are means ± standard error (n = 3). * Significantly different from control (Mann Whitney U test at $p < 0.05$).

3.2 Influence of solar radiation and bacteria on TA and natural DOC

To investigate the influence of solar radiation and bacteria on the allelopathically active TA lake water with and without TA was exposed for three weeks to solar radiation of the full sunlight spectrum in UV permeable plastic bags or kept in the dark with and without bacteria using a full factorial design. The size fractions of chromatographically detectable organic carbon of the different samples were analyzed at the start, after two and three weeks of exposure by LC-OCD (see 2.3.2.1 in *Material and Methods*) to follow structurally changes of TA in lake water by photolytic and microbial degradation. Concomitantly the allelopathic effect of degradation products was tested on phytoplankton growth (*Desmodesmus armatus*).

3.2.1 Effects of solar radiation on natural lake DOC and TA

At the beginning of the experiment addition of TA increased the chromatographically detectable DOC (CDOC) from the bacteria free lake water by 17 % (14.8 mg ± 5% C L^{-1}; -TA, - bacteria treatment; 17.3 mg ± 5% C L^{-1} + TA, -bacteria treatment, Fig 17 A, B). With bacteria (+bacteria) chromatographically detectable DOC was slightly higher (17.6 ± 5% mg C L^{-1}) due to extracellular carbon produced by heterotrophic and autotrophic bacteria, however, the addition of TA did not further increase the chromatographically detectable DOC (Fig.17 C, D). This might be due to initial polymerization processes and aggregation with proteinous substances of the microorganisms (Scalbert 1991) which impeded detection by LC-OCD. After two weeks of exposure to solar radiation the chromatically detectable DOC in lake water with TA reached maximal values (26.1 and 27.2 ± 5% mg C L^{-1}; lake water + TA without and with bacteria, respectively), whereas values in the dark were lower (21 ± 5% mg C L^{-1}), however, did not differ between bacterial treatment.

After three weeks of exposure to solar radiation the chromatographically detectable DOC did not differ for lake water with TA between bacterial treatment (25.9 ± 5% and 26.1 ± 5% mg C L^{-1}, respectively, Fig. 17 B, D). In contrast, the chromatically detectable DOC in dark incubated lake water with TA and bacteria (-light, +bacteria) was significantly lower (18.4 ± 5% mg C L^{-1}) than parallel treatment without bacteria (28.3 ± 5% mg C L^{-1}, Fig. 17 B, D).

Photometrically detected total phenolic compounds (TPC) after reaction with the Folin Reagent confirmed the presence of phenolic substances after addition of TA. In lake water with TA the TPC values were seven (- bacteria) to ten (+ bacteria) times higher than in lake water

without TA. After three weeks of exposure the total phenolic compounds of lake water of all treatments decreased down to values near the detection limit (<1 mg L^{-1}).

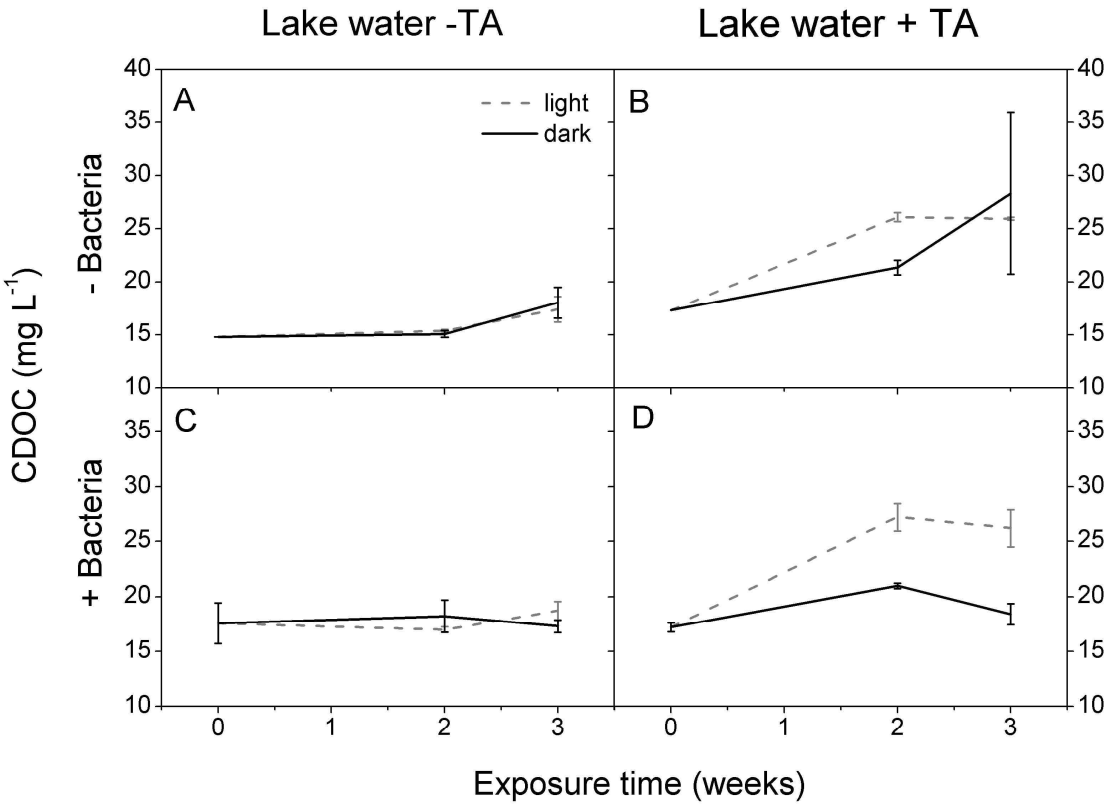


Fig. 17: Concentrations of chromatographically detectable dissolved organic carbon (CDOC means ± SE) in lake water samples exposed to solar radiation (+light) or darkness (-light) with and without tannic acid (+/-TA) and bacteria (+/-bacteria). N=3, Results of ANOVA and post hoc tests are given in Table 9.

Differences between the single size DOC fractions of natural lake water after exposure to sunlight and bacteria were most notable for the humic substances (HS) like compounds after two weeks with minimal values in natural lake water with bacteria and highest values in paralles incubated in the dark (Fig. 18, HS, A, B, Table 11). In contrast, addition of TA to lake water reversed the observed pattern and showed lowest concentration of HS in the dark incubation with bacteria and highest concentrations in samples exposed to solar radiation regardless of bacterial presence (Fig. 18, HS, C, D, Table 11). The specific UV absorbance of HS (SUVA) decreased in the light treatments with and without bacteria compared to the respective dark treatments both in lake water with and without TA (Fig. 18, SUVA, A-D, Table 11). We found increased fractions of HMWS both with and without TA exposed to solar radiation with bacteria at the end of exposure (Fig. 18, HMWS, B, D). The concentration of LMWS in lake water without TA was highest with bacteria in the dark compared to parallel treatments exposed to solar radiation for two weeks (Fig. 18, LMWS, B, Table 11). In lake water with TA, most

LMWS accumulated in the light treatment without bacteria and slightly less with bacteria. However, lowest LMWS concentrations were found in both dark treatments (+ and –bacteria) (Fig. 18, LMWS, C, D; Table 11).

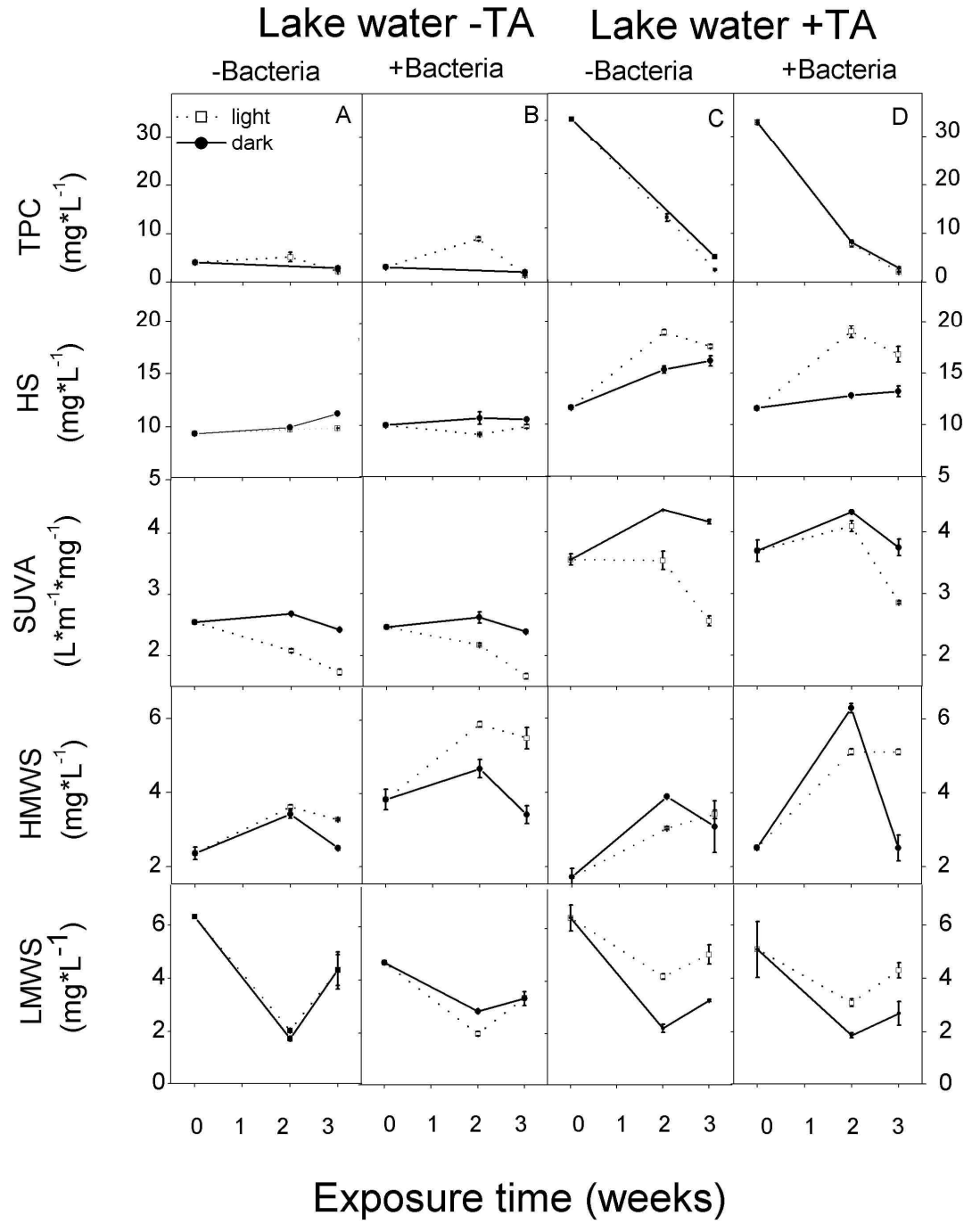


Fig. 18: Concentrations of different size fractions of dissolved organic carbon: humic substances (HS), aromatic substances (SUVA), high molecular weight substances (HMWS), low molecular weight substances (LMWS) and total phenolic compounds (TPC) of lake water samples exposed to solar radiation (+light) or darkness (-light) with and without tannic acid (+/-TA) and bacteria (+/-bacteria). Results of ANOVA and post hoc tests are given in Table 11.

A multiple analysis of variance (MANOVA) separately performed for lake water with and without TA revealed a significant impact of both factors (sunlight, bacteria) on the chromatographically detected DOC and on the different size fractions in the general model for the +TA treatment (Table 10). Whereas bacteria significantly influenced all chemical variables, sunlight only affected HMWS and LMWS (Table 10). HS could not be included because it did not fulfill the test preconditions.

Table 10: Significance (p values) of general model and of single factors (+/-bacteria and +/-light) of MANOVA comparing chromatographic detectable DOC (CDOC), high molecular weight substances (HMWS), humic substances (HS), specific UV absorption (SUVA) of HS and low molecular weight substances (LMWS) as dependent variables separately for lake water with (+TA) and without tannic acid (–TA). Abbreviations: n.c.: not calculated, * prerequisite of normally distribution was not fulfilled.

Treatment	**Dependent variables**	**CDOC**	**HMWS**	**HS**	**SUVA**	**LMWS**
		p	**p**	**p**	**p**	**p**
-TA	Model	0.012	<0.001	0.001	0.055	0.539
	light	n.c.	n.c.	n.c.	n.c.	n.c.
	bacteria	0.012	<0.001	0.001	0.055	0.539
+TA	Model	0.005	<0.001	<0.001	0.004	<0.001
	light	0.002	<0.001	*	0.142	<0.001
	bacteria	0.036	<0.001	*	0.008	<0.001

A one way ANOVA of the combined effects of sunlight and bacteria revealed most significant differences of chromatographically detectable DOC and of its different size fractions including the HS fraction for both factor (bacteria, sunlight) separately performed for lake water with and without TA after two weeks (Fig. 18, +/-light, +/- bacteria; Table 11).

Table 11: Significance (p values) of one way ANOVA and results of post hoc tests (differences between treatments are labelled by different letters in one row) comparing total CDOC and single chemical fractions of DOC as in Table 12 and algal growth rate (μ) between the combined treatments (+/-bacteria, +/-light) separately for +TA and -TA treated lake water after two weeks of exposure. n.c.: not calculated, * 1 prerequisites of normal distribution was not fulfilled *2. test of homogeneity of variance was not fulfilled.

Anova	treatment	CDOC	HMWS	HS	SUVA	LMWS	μ (d^{-1})
p		*2	*1	0.038	<0.001	<0.001	0.007
-TA	+ light, - bacteria			ab	a	a	ab
	+ light, + bacteria			a	a	a	ab
	- light, - bacteria			ab	b	a	b
	- light, + bacteria			b	b	b	a
p		0.006	<0.001	<0.001	*2	<0.001	<0.001
+TA	+ light, - bacteria	ab	a	c		c	c
	+ light, + bacteria	b	c	c		b	a
	- light, - bacteria	a	b	b		a	b
	- light, + bacteria	a	d	a		a	c

Differences in diagensis of lake water CDOC with TA and bacteria between light and dark treatments can be followed when comparing the chromatographic spectrum of DOC at the beginning and at the end of exposure (Fig. 19, t_0-t_2). At the beginning of the exposure time (t_0) the chromatogram of CDOC showed an enhanced size fraction at retention time (RT) 20 - 30 min (HMWS) with bacteria compared to bacteria free water samples. This was consistent for both treatments with and without TA at the start and at lower concentrations of HMWS at the end of exposure (Fig. 19 B, D, F, H). TA treated lake water showed higher LWMS and an elevated level of all CDOC size fractions after addition of TA (Fig. 19, AB-CD). At the end of exposure (Fig. 20 E-F), we observed photolytic degradation of LMWS in the absence of bacteria in untreated lake water (-TA; Fig. 19 E). Bacterial presence in lake water with and without TA resulted in higher concentrations of HMWS, elevated even in the TA treatment exposed to solar radiation. In contrast bacteria significantly decreased concentrations of HS and LMWA in the dark (Fig. 19 E-F, G-H). When comparing chromatograms of lake water with TA and bacteria from the start and the end of exposure the DOC spectrum showed lower levels for all size fractions, especially for HS in the dark. Thereby, the chromatogram of the

light treatment decreased less over the whole DOC spectrum and displayed a well established HS fraction (Fig. 19 D, H).

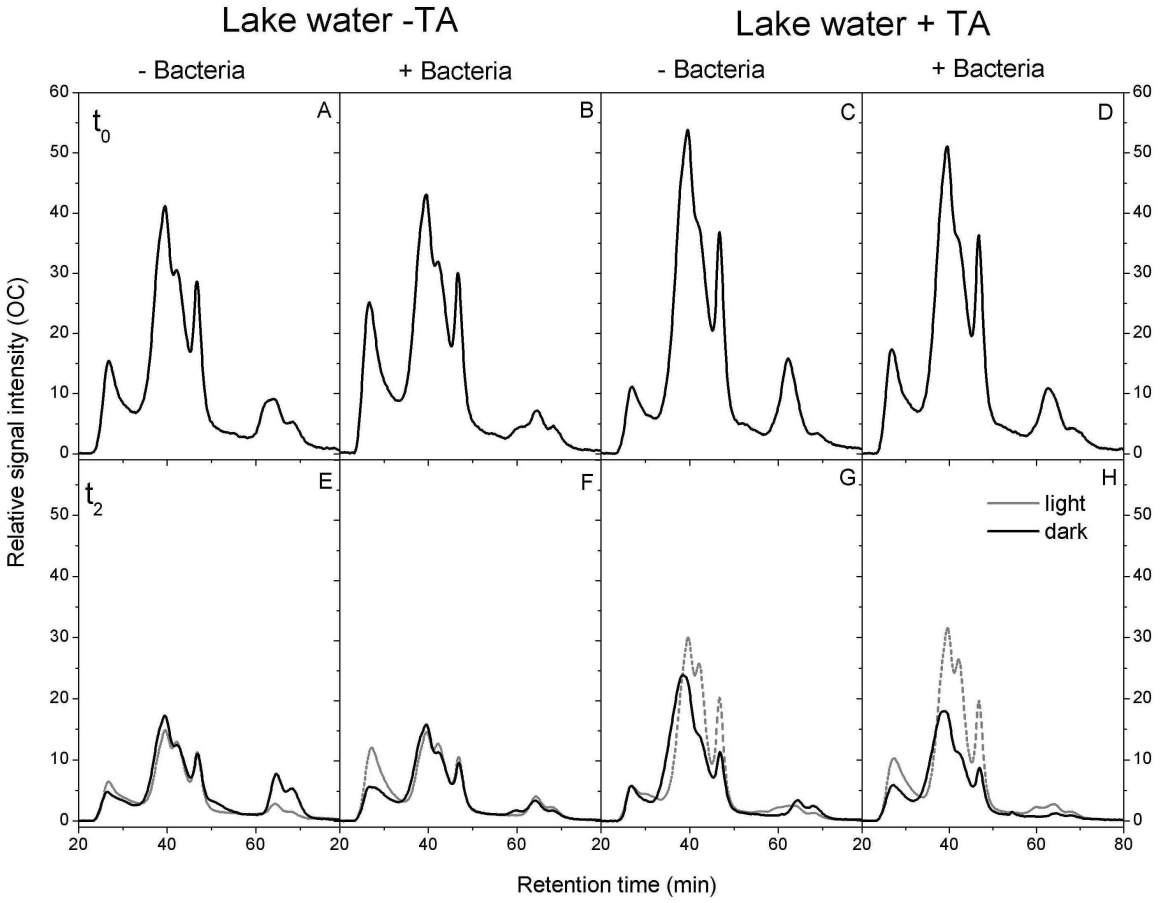


Fig. 19: Influence of solar radiation and bacteria on chromatographic size distribution of phenolic substances (OD_{280}) in lake water with and without additional tannic acid (TA). Comparison of samples of water without TA (lake water –TA) and samples containing TA (lake water +TA) before (t_0: A-B) and after (t_2: E-F) the exposures to solar radiation or dark incubation with (+bacteria) and without bacteria (-bacteria) see Material and Methods chapter 2.3.2). Only one replicate is shown for clarity.

3.2.2 Influence of natural DOC and TA degradation products on *D. armatus*

Growth of D. armatus was strongly inhibited cultured in lake water with TA prior to exposure (Fig. 20 t0 B, D) compared to growth of the control in natural lake DOC (Fig. 20 t0 A, C). If grown in two (t1) and three weeks (t2) of incubation D. armatus performed nearly identical growth response cultured in lake water of the respective treatments. Comparison between treatments by one way ANOVA performed separately for +/-TA revealed lowest algal growth if cultured in lake water without TA that has been exposed in the dark with bacteria. Moderate algal growth was recorded if cultured in lake water without TA previously exposed to solar radiation +/-bacteria. Algal grew best in lake water without TA and bacteria in the dark (Table

11, Fig. 20 A-C, F = 8.6, p = 0.007). Algae grown in lake water with TA previously exposed to solar radiation and bacteria performed lowest growth in the TA treatment. Algae grew better if grown in lake water with TA with bacteria in the dark or if cultured in lake water with TA exposed to solar radiation without bacteria (Table 11, ANOVA, F = 42.4, p < 0.001, Fig. 20 B, D).

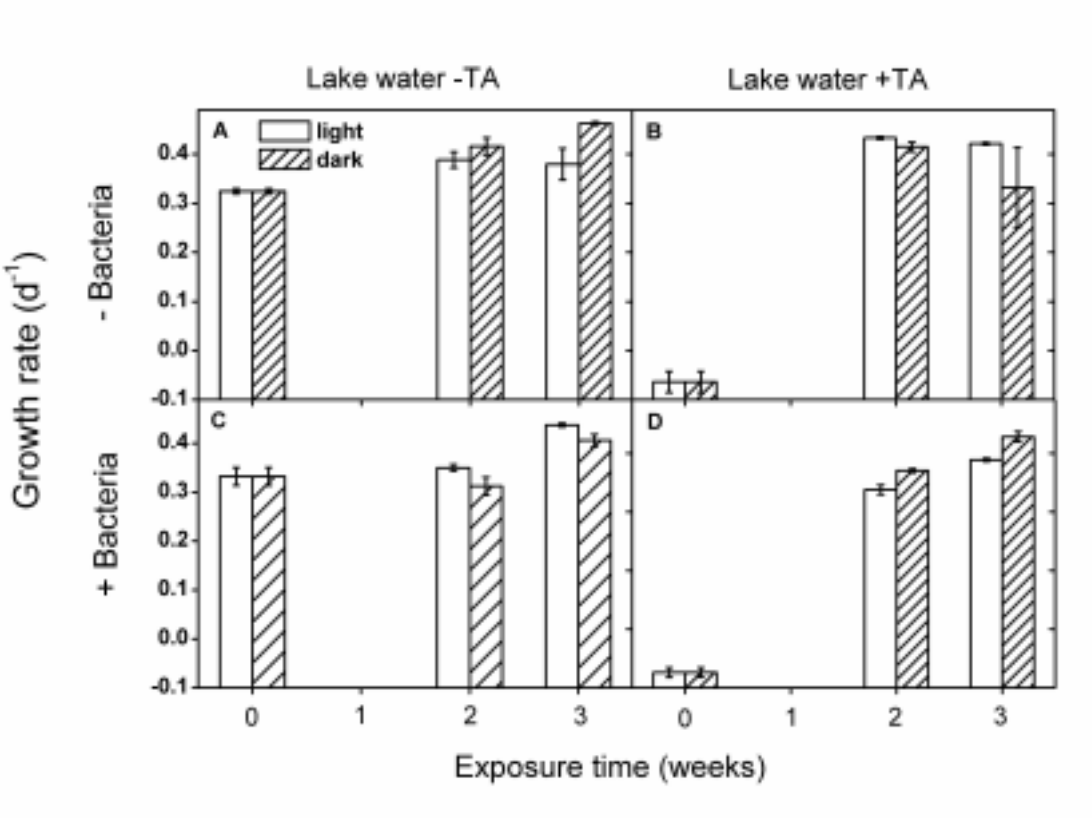


Fig. 20: Growth rates of the green alga *Desmodesmus armatus* L. (start concentration 46 µg chl *a* L^{-1}) cultivated in nutrient enriched lake water (see 2.3.6 and 2.3.8 *Material and Methods*) that has been exposed with and without tannic acid (TA) to solar radiation or in the dark with and without bacteria. Results of ANOVA and post hoc tests are given in Table 10.

3.3 Influence of temperature on allelopathic effects of TA on phytoplankton

To investigate the influence of temperature on the allelopathic effect on phytoplankton growth a pelagic and a benthic diatom as well as a chlorophyte were cultured at three temperatures (10, 15, 20°C) and temperature dependent growth response to tannic acid (TA) was compared to growth rates of untreated controls. Parallel approaches with (xenic) and without (axenic) bacteria were performed with the benthic diatom and the green alga to investigate if bacteria influence algal response to TA dependent on temperatures.

3.3.1 Algal growth rate and bacteria to algae ratio

Growth rate based on ground fluorescence measurements of the pelagic diatom *Stephanodiscus minutulus*, a representative of spring phytoplankton was highest at lowest temperature (10°C) without TA addition and decreased with increasing temperature (Fig. 21 A, $F = 690$, $p < 0.001$). Addition of TA always inhibited algal growth with maximal inhibition at 15 °C, less at 10 °C and lowest inhibition of growth rate at 20 °C compared to growth rates of control cultures grown at respective temperatures (Fig. 21 A, $p = 0.018$, $F = 8.4$). Algae cell counts starting from $t_0 = 1.85\ 10^5 \pm 2.02\ 10^4$ mirrored growth rate after four days, however, differed not significantly between different temperatures due to high variability (Fig.21 B, control: $p = 0.044$, $F = 5.5$; TA treatment $p = 0.045$, $F = 5.4$).

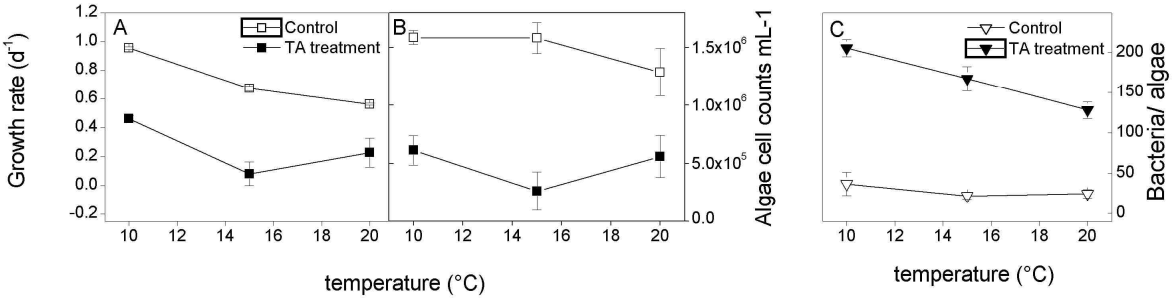


Fig. 21 Growth rate (A), algae cell counts (B) and bacteria/ alga ratio (C) of the pelagic diatom *Stephanodiscus minutulus* with bacteria in culture treated with daily addition of 1µg TA (TA treatment) or with A. dest (control) at different temperatures (10, 15, 20°C). Error bars are the confidence intervals of the three replicates ($\alpha = 0.05$).

The bacteria to algae ratio was always higher in algal cultures *Stephanodiscus minutulus* treated with TA than in parallel controls indicating that TA addition seems to support bacteria (Fig. 21 C). Furthermore, the bacteria to algae ratio increased with growth rate at lower tem-

perature indicating that bacteria mainly depend on exudates of photosynthetic active algae and to less extent on degraded cells or degradation products.

The growth rate of the benthic diatom *Gomphonema parvulum* without TA and bacteria (control) was highest at 20°C and lowest at 10°C in the (Fig. 22 A, $F = 337.4$, $p < 0.001$). If treated with TA, the growth of the axenic diatom was maximal at 15°C, moderate at 10°C and lowest at 20°C (Fig. 22 A, $F = 66$, $p < 0.001$). Compared to the untreated control TA treatment increased algal growth at 10 and 15 °C, whereas algal cultures grown at 20°C with TA were inhibited compared to parallel controls. The xenic algal culture without TA performed comparable growth response pattern to TA treated axenic algal culture with maxima at 15 °C and comparable lower growth rates at 10 and 20 °C (Fig. 22 B, $F = 19.8$, $p = 0.002$). TA addition to the xenic benthic diatom decreased algal growth compared to untreated controls at 10 °C, and enhanced algal growth at 15°C and 20 °C (Fig. 22 B, $F = 424.3$, $p < 0.001$). Algal cell counts in bacteria free controls surpassed the TA treated algae at 15 and especially increased at 20°C compared to TA treated algal cell counts. With bacteria in algal culture the cell counts of the controls always were lower than in TA treated cultures of *G. parvulum*.

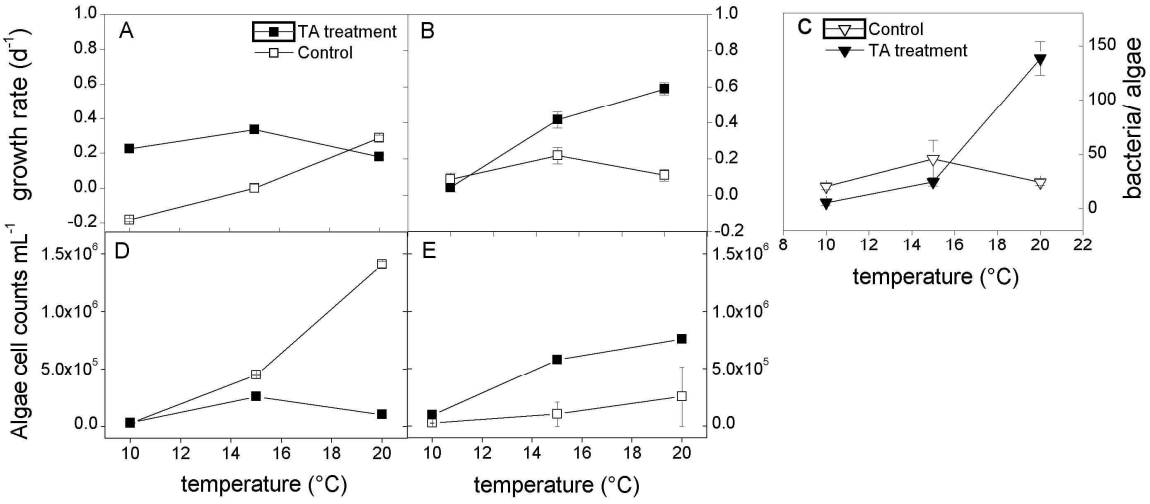


Fig. 22 A: growth rate of the benthic diatom *Gomphonema parvulum* without bacteria, B: growth rate of *G. parvulum* with bacteria in culture treated with (TA treatment) and without tannic acid (Control), C: bacteria/ alga ration and D: algal cell counts of axenic and E: xenic of *G. parvulum* at different temperatures (10, 15, 20°C). Error bars are the confidence intervals of the three replicates ($\alpha = 0.05$).

Bacteria to algae ratio in cultures of the benthic diatom *Gomphonema parvulum* followed the algal growth rate indicating microbial use of algal derived substrate. Bacterial abundance in TA treatment surpassed the control at 20 °C indicating that bacteria profiteered from TA treatment at higher temperatures and were discriminated against algae at lower temperatures.

The growth rate of *Desmodesmus armatus* was generally less affected by TA at all temperatures although a slightly higher concentration of TA was applied (2µL TA L^{-1}). The bacteria free culture of *D. armatus* in both (+/- TA) treatments performed maximal growth at 15°C, moderate growth at 20°C and lowest growth at 10°C (Fig. 23 A, Chi Square = 7.5, p = 0.024), With bacteria algal growth of the control was maximal at 20°C decreasing with descending temperature to growth minimum at 10 °C (Fig. 23 B, F = 162.6, p < 0.001). Treatment with TA increased algal growth of axenic *D. armatus* at low temperature, did merely affect growth of the green alga at 15°C and inhibited growth at 20°C compared to control (Fig. 23 A, t-test, p < 0.001, p = 0.08, p = 0.002 respectively). Xenic algal cultures treated with TA were inhibited at 20°C, slightly enhanced at 10°C and 15°C (Fig. 23 B, t-test, p = 0.001, each). Algal cell counts in axenic cultures deviated from fluorescence based growth rate at 15 °C with clearly enhanced algal abundance and decreased cell counts at 20 °C (Fig. 23 D), whereas in cultures with bacteria we observed similar results to algal growth rate based on fluorescence measurements.

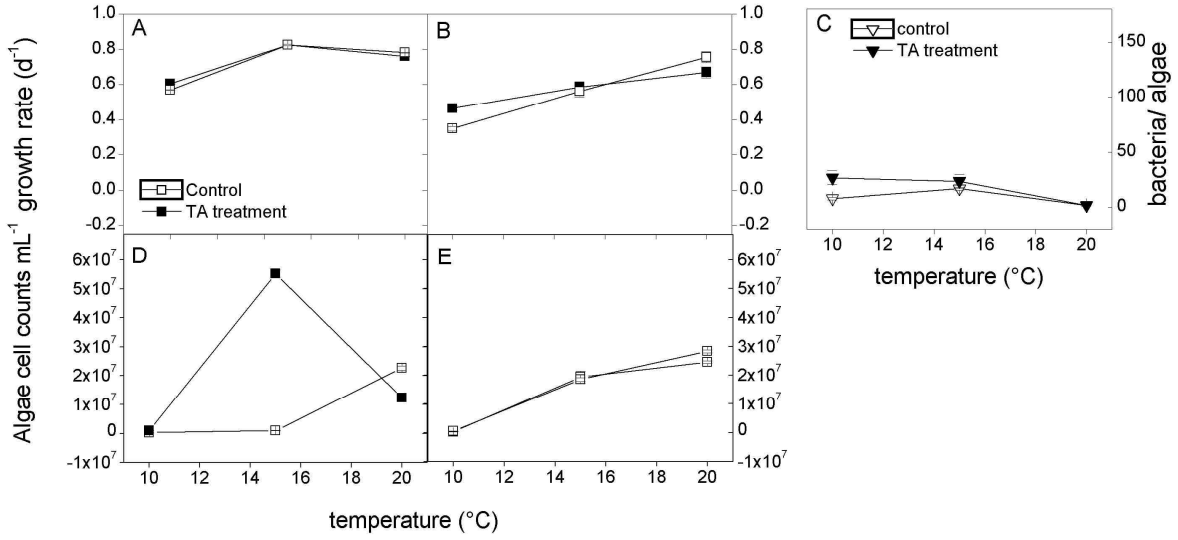


Fig 23 A: growth rate of the green alga *Desmodesmus armatus* without bacteria, B growth rate of *D. armatus* with bacteria in culture treated with (TA treatment) and without tannic acid (control), C: bacteria/algae ratio and D: algae cell counts of axenic algal culture and *D. armatus* with bacteria at different temperatures (10, 15, 20°C). Error bars are the confidence intervals of the three replicates (α = 0.05).

The bacteria to algae ratio was higher in the TA treatment compared to bacteria to algae ration of controls cultures cultivated at 10° C and at 15°C (Fig. 23 C, U-test, p = 0.036, F = 411.2, p < 0.001), indicating that bacteria in culture with *Desmodesmus armatus* profiteered of TA treatment at these temperatures. However, at maximal growth of the xenic green algae at 20°C

the bacteria to algae ratio with and without TA treatment were not significantly different (U-test, $p = 0.487$), indicating that TA did not affect the quantitative relation of algae and bacteria at maximal algal growth for this species.

3.4 Influence of different specialized bacteria and initial algal concentration

To investigate the influence of bacteria of different origin (unspecific bacteria from the culture solution and specific bacteria from the algal culture) and different initial algal concentrations on the growth response of algae to allelopathic active tannic acid (TA) parallel approaches with *Desmodesmus armatus* at low initial algal concentration (experiment 1, 7.8 mg Chl-*a*/m^3) and at double initial concentration (experiment 2, 14.7 mg Chl-*a*/m^3) were compared. The growth parameter of the green algae after culture in bacteria free and with and without TA in the absence and presence of the different specialized bacteria is presented in table 12 for both experiments. The algal growth response to TA plotted as inhibition in percent of the untreated control and the bacteria to algae ratio in percent of respective controls are presented in Fig. 24.

3.4.1 TA effect on algal growth (*Desmodesmus armatus*)

In both experiments 1 and 2 TA treatment caused significantly lower growth rate (μ), maximal fluorescence (Fm) and effective quantum yield of PSII (Fv/Fm) for the different cultured algae (bacteria free, cultures with unspecific and specific bacteria) compared to the respective controls (Table 12; Student's t-test, $p < 0.01$). Differences were also found in all algal cultures for biovolume dependent on TA treatment but not for the highly variable response of the axenic algae in experiment 2 (Table 12, Student's t-test, $p = 0.43$). The chosen TA concentration was therefore suitable to mimic the inhibitory effect of allelochemicals exuded from *Myriophyllum* species on the growth and photosynthesis of phytoplankton (Gross et al. 1996; Körner und Nicklisch 2002).

Table 12: Algal growth parameter (± SE) at the end of exposure to tannic acid (TA): growth rate, biovolume, maximale fluorescence (Fm) and the effective quantum yield of PSII, the quotient of variable and maximale fluorescence (Fv/Fm), bacteria/algae ratio of the different alga cultures of *Desmodesmus armartus* without bacteria (alga – bacteria), with unspecific (alga + unspecific bacteria), specific (alga + specific bacteria) and with and without TA treatment (+/-TA) each.* stained fragments/dead bacteria were counted as bacteria but did not further proliferate

Algal growth parameter	**growth rate (d^{-1})**	**Biovolume (nl)**	**Fm**	**Fv/Fm**	**Bacteria /algae**
low algal start concentration (7.8 mg Chl-*a*/m^3)					
Alga – bacteria	0.78 ± 0.02	36.4 ± 2.56	3.34 ± 0.11	0.75 ± 0.001	5.3 ± 5.3*
Alga – bacteria +TA	0.6 ± < 0.001	18.17 ± 0.93	1.05 ± 0.02	0.68 ± 0.004	5.3 ± 5.3*
Alga + unspecific bacteria	0.82 ± 0.001	49.17 ± 1.38	6.11 ± 0.06	0.75 ± 0.001	494 ± 40.8
Alga + unspecific bacteria +TA	0.65 ± 0.01	33.56 ± 1.7	2.14 ± 0.05	0.69 ± 0,002	736 ± 59.1
Alga + specific bacteria	0.75 ± 0.01	48.74 ± 3.97	4.83 ± 0.06	0.75 ± 0.002	778 ± 84.7
Alga + specific bacteria +TA	0.61 ± 0.001	34.95 ± 3.73	1.9 ± 0.01	0.68 ± 0.001	805 ± 71.2
high algal start concentration (14.7 mg Chl-a/m3)					
Alga – bacteria	0.72 ± 0.01	39.25 ± 9.4	4.93 ± 0.14	0.75 ± 0.002	0
Alga – bacteria +TA	0.59 ± 0.01	31.13 ± 4.39	2.13 ± 0.04	0.7 ± 0.001	0
Alga + unspecific bacteria	0.75 ± 0.01	63.25 ± 4.8	5.93 ± 0.09	0.72 ± 0.005	113 ± 39.6
Alga + unspecific bacteria +TA	0.63 ± 0.02	38.9 ± 1.8	3.03 ± 0.15	0.71 ± 0.002	250 ± 41.3
Alga + specific bacteria	0.76 ± 0.01	78.4 ± 4.16	6.58 ± 0.21	0.75 ± 0.003	238 ± 37.3
Alga + specific bacteria +TA	0.6 ± 0.02	35.49 ± 0.33	2.28 ± 0.14	0.68 ± 0.004	767 ± 112

3.4.2 Influence of initial algal concentration on algal growth response

The results from both experiments are presented as inhibition in percent of difference of algal growth response treated with TA compared to respective untreated control (Fig. 24). In the experiment 1 (low initial algae concentration) the growth rate, biovolume and maximale fluorescence were maximal lowered by TA in bacteria free cultures of *D. armatus* (note that inhibition is plotted positively in Fig. 24). Xenic algal cultures containing unspecific bacteria from culture solution moderately weakened the inhibitive effect of TA on the green algae. Lowest inhibition of algal growth by TA was observed in algae cultures containing specific bacteria from the algal culture (Fig. 24, A1, B1, C1; ANOVA: Table 13). In contrast, in experiment 2, algae growth rate, biovolume, maximale fluorescence and effective quantum yield of PS II (F_v/F_m) were maximal inhibited by TA in algal cultures with specific bacteria and less affected by TA with unspecific bacteria and without bacteria (Fig. 24, A2, B2, C2, D2; ANOVA: Table 13).

Table 13: Results of multiple comparisons between growth rates (μ), maximal fluorescence yield (Fm), effective quantum yield (Fv/Fm), biovolume and bacteria/alga ratios of untreated controls (Fig. 24 A-E 1, 2) and per-cent inhibition after tannic acid treatment (Fig. 25 A-E 1, 2) of axenic cultures, cultures with suspended bacteria and xenic cultures of *Desmodesmus armatus* in 2 experiments with different initial algae biomass using one-way ANOVA.

		Controls		**Inhibition**	
		F	***P***	**F**	***p***
μ	**Exp. 1**	60.2	< 0.001	28.9	0.001
	Exp. 2	6.6	0.031	6.9	0.028
Fm	**Exp. 1**	196.5	< 0.001	31.1	0.001
	Exp. 2	19.4	0.002	11.2	0.009
Fv/Fm	**Exp. 1**	4.7	0.06	3.9	0.081
	Exp. 2	11.7	0.01	11.7	0.010
Biovolume	**Exp. 1**	5.5	0.031	5.3	0.048
	Exp. 2	9.1	0.015	9.0	0.016
Bacteria/alga	**Exp. 1**	51.7	< 0.001	9.9	0.013
	Exp. 2	14.3	0.05	10	0.012

3.4.3 The effect of TA on bacteria to algae ratio and bacterial influence on growth response of *Desmodesmus armatus* to TA

Bacterial influence on the effect of TA on the tested green alga differed between the two experiments performed with different initial algae concentrations. High bacteria to algae ratio at low initial algal concentration (exp. 1) indicated a high bacterial influence in algal cultures with unspecific bacteria but not in cultures with specific bacteria that even more lowered algal growth inhibition of TA (Fig. 24 E1; ANOVA: Table 13).

At high initial algal concentration (exp. 2) the increased bacteria to algae ratio after TA treatment coincided with the aggravating bacterial effect of unspecific and specific bacteria increasing the inhibiting effects on fluorescence based growth parameter and biovolume (Fig. 24, A2, B2, C2, D2, E2; ANOVA: Table 13).

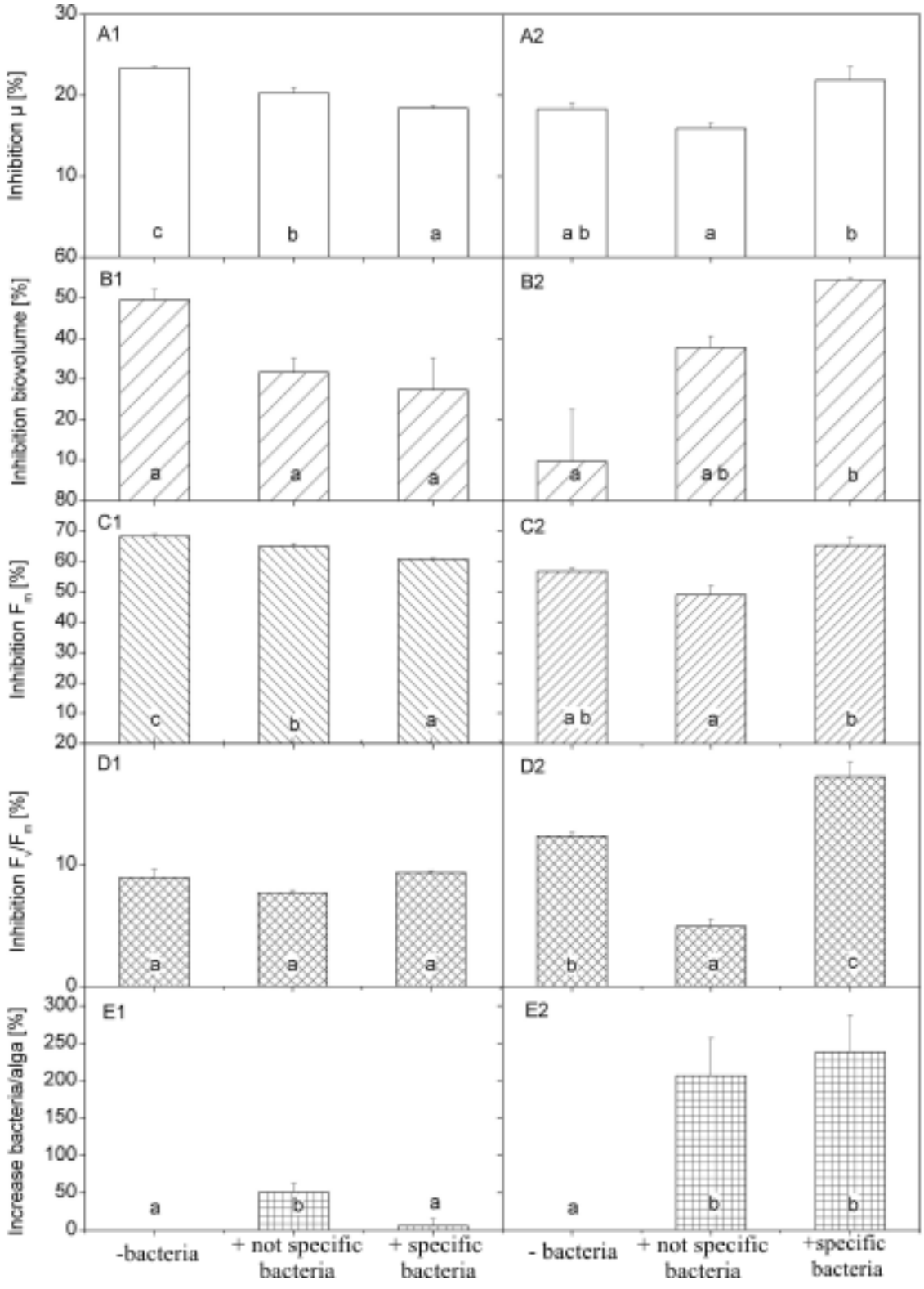


Fig. 24: Inhibition of growth rate (μ) in percent, maximal fluorescence yield (F_m), effective quantum yield (F_v/F_m), biovolume and increase of bacteria to algae ratio of bacteria free cultures of *Desmodesmus armatus* (- bacteria), algal culture with added not specific bacteria from culture solution (+ not specific bacteria) and *D. armatus* with bacteria co-cultivated in algal culture for several generations (+ specific bacteria) treated with tannic acid compared to untreated controls after five days of growth. Multiple comparisons were performed using one way ANOVA and subsequent Tukey's post hoc test (Table 13). Different letters indicate significant differences at $p < 0.05$. Error bars are the standard error intervals of the three replicates.

3.5 Influence of bacterial community composition on the allelopathic effect

In order to investigate if qualitative differences in bacterial community composition, changed by previous exposure to the allelochemical tannic acid (TA), may influence algal growth response to TA, we combined three experiments (so called *phases*, see 2.3.3.3 *Materials and Methods*, Fig. 9). First a natural bacterial community was pre-conditioned to no (0%), low (0.05%) and high concentrations of TA (0.5% TA in solution) to select for bacteria resistant to TA and capable to use TA as substrate (*phase 1*). The different bacteria communities - derived from a natural bacterial community of a humic rich lake (Krumme Laake, see 2.2 *Material and Methods* for physicochemical properties) with a monostand of *Myriophyllum verticillatum.* The pre-conditioned bacteria were cultured together with the axenic diatom *Stephanodiscus minutulus* and algal growth response to daily addition of 1µg TA L^{-1} was compared to parallel bacteria free algal response (*phase 2*). Finally bacteria isolated from the pre-treated bacterial community were tested for their ability to degrade TA and degradation products were tested on changes in growth response (*phase 3*). To follow changes in bacterial community composition molecular-biological approaches such as DGGE and sequencing were used to characterize bacterial community composition of different TA treatments and identify new emerging bacterial strains (see 2.3.4 *Material and Methods* for detailed methodology).

3.5.1 Phase 1 (Pre-treatment of the bacteria with TA)

3.5.1.1 Effects of the allelochemical on the bacteria

Bacterial growth rates were not influenced during pre-treatment with TA (ANOVA, $p > 0.05$ for all treatments, Table 14). Abundance of living bacteria at the end of the pre-treatment, however, was significantly higher in the high TA treatment ($1.5 \pm 0.1 \times 10^6$) as compared to the low TA treatment ($0.9 \pm 0.02 \times 10^6$) (ANOVA, $p = 0.001$, $F = 27.5$). We clustered the DGGE banding patterns of *phase 1* and *2* to follow changes in BCC between both phases (but separately for attached and free-living bacteria). Cluster analysis of free-living bacteria revealed sub-clusters for both phases and sub-clusters between low and high TA treatment (Fig. 25A, H, L). These clusters were clearly different from the control (Fig. 25A, C). BCC of attached bacteria formed a sub-cluster with C and L of ***phase 1*** (Fig. 25B) which was clearly different from that of the high TA addition (H)**.** The DGGE band numbers of attached bacterial communities increased successively from no, low and high TA treatments.

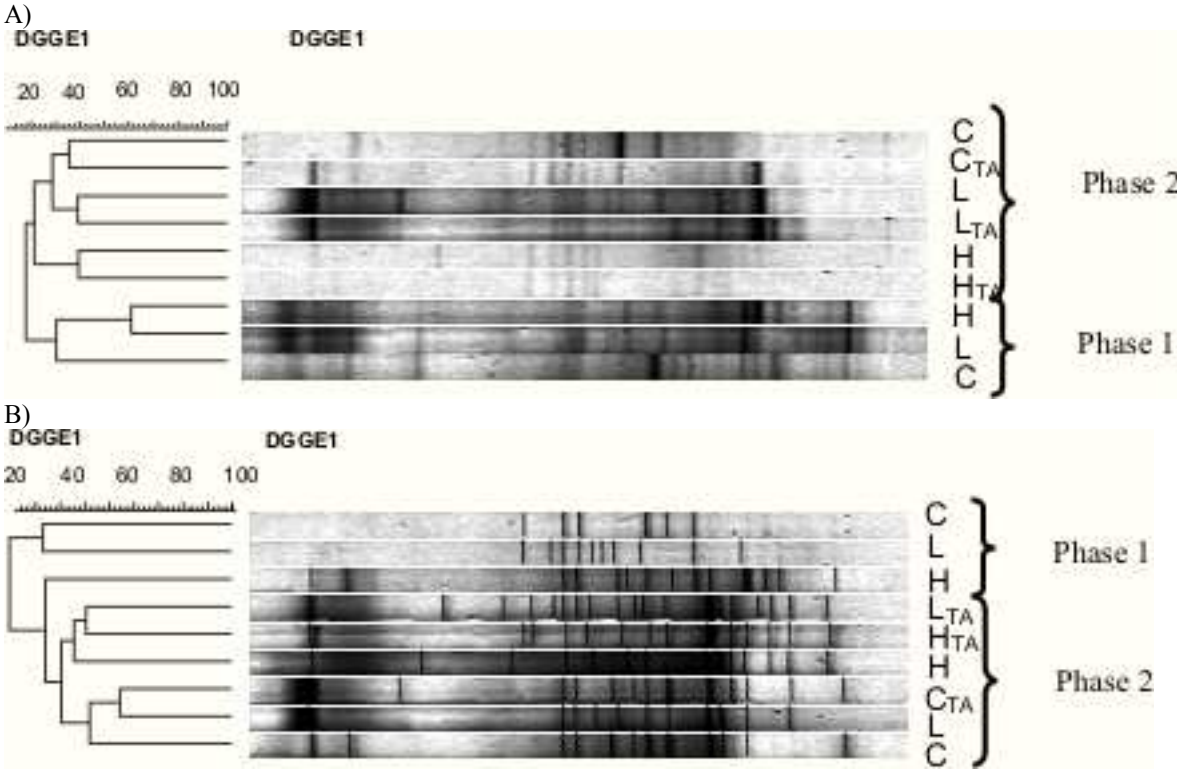


Fig. 25: Dendrogram of cluster analysis of DGGE banding patterns: A) free-living and B) attached bacteria. C, L, H: pre-treated bacterial community of phase 1 (Fig. 10) to no, low (0.05%) and high (0.5%) tannic acid (TA) concentrations. C_{TA}, L_{TA}, H_{TA} refer to respective bacterial communities of phase 2 cultivated with *Stephanodiscus minutulus* with daily TA addition (1 mg l^{-1}) and without further TA addition: C, L, H (Fig.10).

DGGE bands which showed a new or representative emergence in the different pre-treatments were selected. These bands were identified on the basis of alignment of their partial 16S rDNA sequences with closely related sequences of the NCBI gene bank (March, 2009). The results are presented in a phylogenetic tree (Fig. 26) and in Table 15 (Appendix). Attached bacteria of the untreated samples (C) of *phase 1* are characterized by acidophilic Betaproteobacteria such as *Acidovorax sp*. and *Rhodoferrax ferrireducens* (Comamonadaceae). In the free-living bacteria fraction we found potential pathogens such as *Legionella sp.* (Gammaproteobacteria). For further analysis of bacterial community we focussed on attached bacteria, because bacteria-mediated effects on phytoplankton sensitivity to allelochemicals are assumed to be stronger in this fraction. In *phase 1*, additions of low concentration of TA (L) served as an additional carbon source and shifted the BCC towards a more diverse community including Betaproteobacteria of the Burkholderiales, e.g. *Limnobacter sp.,* and Alphaproteobacteria, e.g. *Rickettsia canadensis* and *Rhodobacter sphaeroides*. Additions of high concentration of

TA (H) resulted in the occurrence of Actinobacteria (*Streptomyces*) and Betaproteobacteria of the Comamonadaceae (*Rhodoferrax ferrireducens*).

3.5.2 Phase 2 (*S. minutulus* cocultured with pre-treated bacteria)

3.5.2.1 Effects of TA and Stephanodiscus minutulus on BCC

Bacterial abundance increased during cultivation with *S. minutulus* (*phase 2*, Table 14). At the end of *phase 2***,** minimal bacteria abundance was recorded in the low TA pre-treatment (L; $0.66 \pm 0.06 \times 10^7$ cells mL^{-1}) and maximal abundance was found for the high TA pre-treatment without further TA addition (H; $0.8 \pm 0.09 \times 10^7$ cells mL^{-1}). In *phase 2* cultured together with the diatom and further TA addition bacterial abundance was lowest in the high TA pretreated bacteria cocultured with S. minutulus (H_{TA}; 1.1 ± 0.36 cells mL^{-1}) and highest in the previously untreated natural bacterial community (control) treated with daily TA addition in phase 2 (C_{TA}; $1.36 \pm 0.25 \times 10^7$ cells mL^{-1}). The average bacterial growth rate over all bacterial communities in *phase 2* was reduced (0.31 ± 0.02 d^{-1}) to almost half of the overall growth rate of bacteria in *phase 1* (0.61 ± 0.01 d^{-1}). Growth rates of living and total bacteria did not differ between treatments with further TA addition (Table 14, ANOVA, $p > 0.05$). However, living bacteria without further TA addition and pre-treated with the high TA concentration revealed a significantly higher growth rate (0.34 d^{-1}) as compared to C and L (0.19 and 0.25 d^{-1}, respectively) (Table 14, ANOVA, $p = 0.004$, $F = 16.59$). This pattern is consistent with the cluster analysis of the respective DGGE banding pattern of attached bacteria and free-living bacteria exposed to TA in the presence of *S. minutulus* grouped into TA concentration dependend distinct sub-clusters (see 3.5.1.1.).

Table 14: Growth rates (means with standard error; SE) of living bacteria and total bacteria during *phase 1* pre-treated with no (C=control), low (L = 0.05%) and high (H = 0.5%) of tannic acid (TA) and in co-culture with *S. minutulus* (*phase 2*) with and without daily addition of 1µgL^{-1} TA. Significant differences in growth rates between pre-treatments were indicated with bold numbers and assigned with low case letters that refer to $p < 0.001$, tested with ANOVA and subsequent Tukey-B posthoc test.

Experimental phase/ Pre-treatment (L;C;H)		**Growth rate (d^{-1}) ± SE**			
		Living bacteria	**Total bacteria**	**Living bacteria (TA-treatment)**	**Total bacteria (TA-treatment)**
Phase 1: pre-treatment with TA	**C**	0.63 ± 0.003	0.64 ± 0.012		
	L	0.65 ± 0.013	0.61 ± 0.027		
	H	0.59 ± 0.011	0.56 ± 0.014		
Phase 2: co-incubation with algae	**C**	0.19 ± 0.027 (a)	0.26 ± 0.02	0.36 ± 0.043	0.30 ± 0.04
	L	0.25 ± 0.014 (a)	0.28 ± 0.01	0.48 ± 0.037	0.38 ± 0.03
	H	0.34 ± 0.012 (b)	0.26 ± 0.005	0.36 ± 0.05	0.27 ± 0.05

Amongst bacteria originating from the untreated control of *phase 1* and later treated with TA together with *S. minutulus* in *phase 2* (C_{TA}), Betaproteobacteria related to *Delftia acidovorans* SPH-1 were found. When pre-treated with low TA concentrations (L and L_{TA}) Alphaproteobacteria (e.g. *Erythrobacter litoralis*) occurred. In pre-treatment with high TA concentrations and cultured together with the diatom (H and H_{TA}) bacteria affiliated with *Actinobacteria* closely related to an uncultured clone (AJ575542) of humic Lake Grosse Fuchskuhle (Burkert 2003; Allgaier 2006) were found (Fig. 26). *Rhodoferax ferrireducens* (Burkholderiales, Comamonadaceae) occurred in the bacterial community pre-treated with high TA concentrations (H) and also in the untreated bacterial community (C).

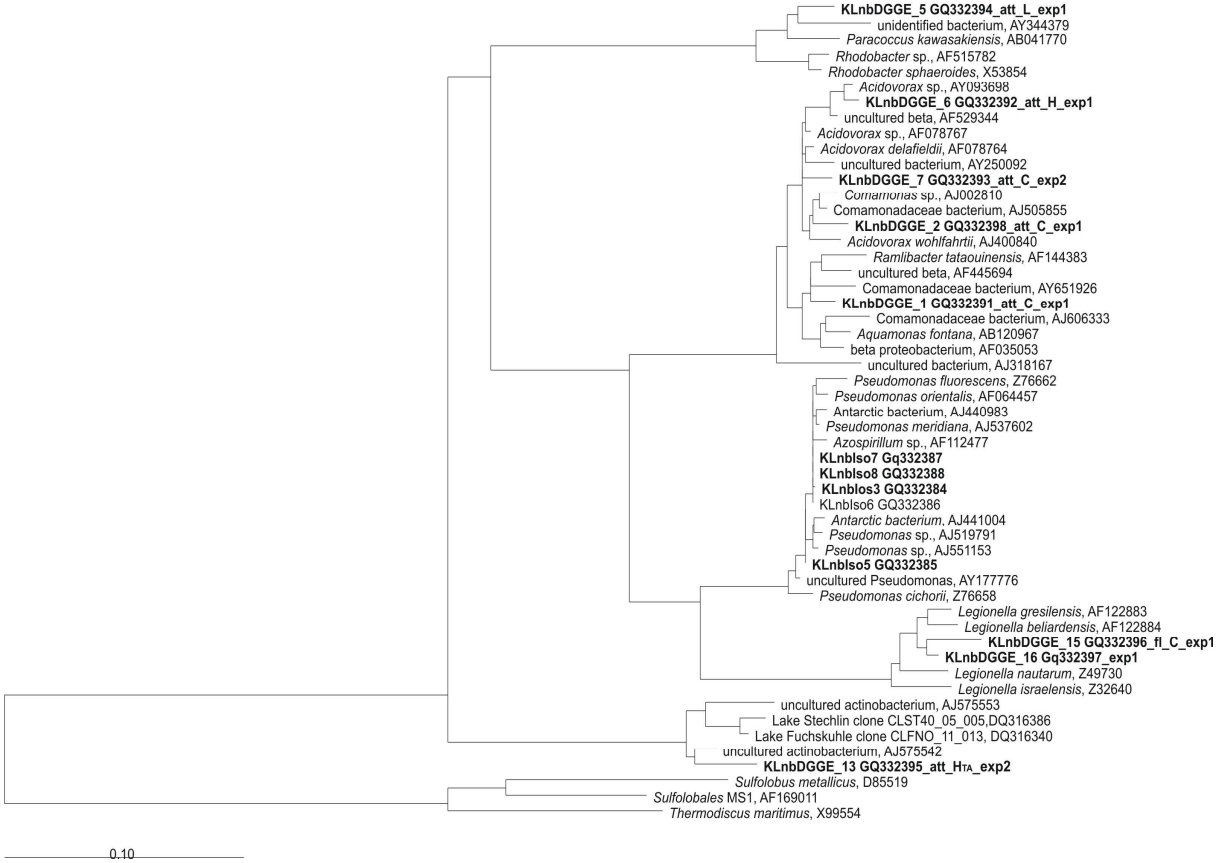


Fig. 26: Phylogenetic (neighbour joining) tree based on 16S rDNA sequences showing the relationship of representatives of DGGE bands of attached (att.) and free living bacteria (f.l.) with different tannic acid addition during pre-treatment (phase 1, C: no, L: low and H: high TA concentrations) and of the sensitivity test without further TA (phase 2, C, L, H) and with further TA addition (C_{TA}, L_{TA}, H_{TA}). The phylogenetic affiliation of sequenced DGGE bands (indicated by labels for DGGE and gene bank accession number) and isolates (indicated by the label "Iso") of Lake Krumme Laake enriched with TA as the sole carbon source is given in Table 15 in the Appendix.

3.5.2.2 Bacterial influence on allelopathic effects on Stephanodiscus minutulus

Effects of specific bacterial communities on the sensitivity of *S. minutulus* to TA were detected by comparing growth rates of axenic and xenic *S. minutulus* after TA addition. Growth rates based on biovolume and cell counts gave the same tendency as growth rates based on F_0 but reacted with a time delay and, therefore, are not presented. Growth rates (based on F_0) of axenically cultured *S. minutulus* incubated with bacteria free filtrates pre-treated with high TA concentration were strongly inhibited resulting in negative growth rates regardless of further treatment with TA (Fig. 27 C, D; H and H_{TA}). Growth rates of axenic diatoms cultured with bacteria free filtrates of the untreated natural bacterial community (control) and of the bacterial community exposed to low TA concentration were both equally positive regardless of further daily TA treatment during algal growth experiment (*phase 2*; Fig. 27 C, D; ANOVA, $p <$

0.001, F = 22.6). In contrast, bacterial cultures previously exposed to high TA concentrations without further daily TA addition (H) increased xenic algal growth compared to algal growth with bacteria not pre-treated with TA (C) or with low TA (L) (ANOVA, p = 0.001, F = 26.3, Fig. 27A). Further addition of TA to xenic algal cultures with bacteria pre-treated with high (H_{TA}) reduced growth of *S. minutulus* compared to algal cultures with bacteria not previously treated with TA (C_{TA}) was not significantly different from algal growth with bacteria pre-treated with low TA concentrations (L_{TA}) (ANOVA, p = 0.01, F = 11, Fig. 27B).

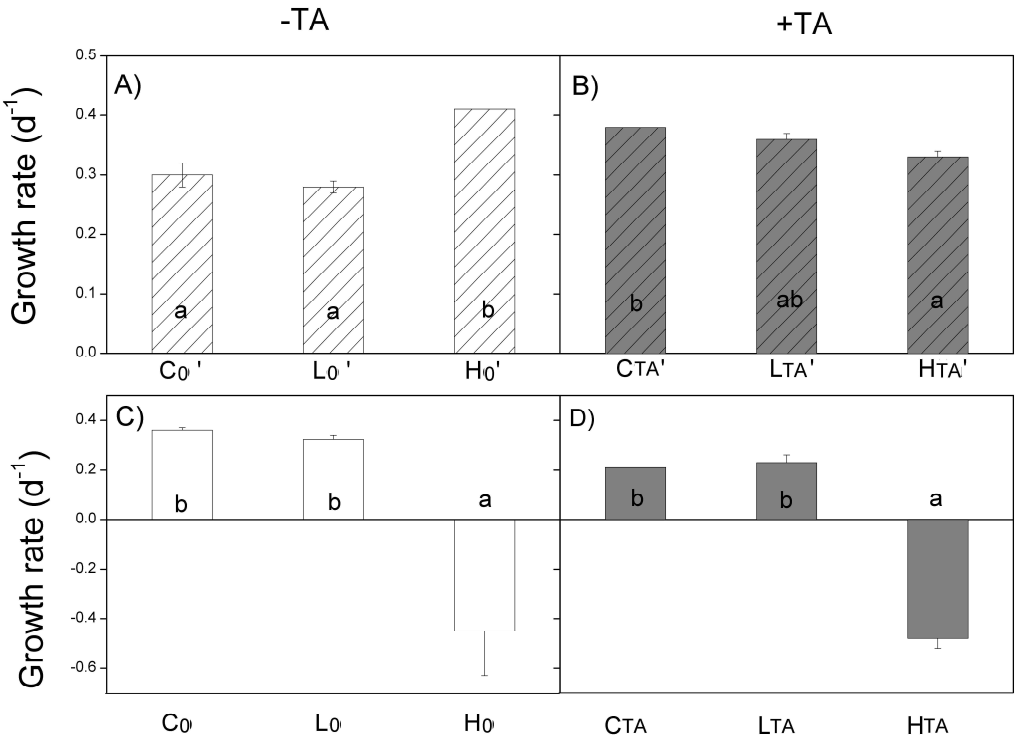


Fig. 27: Growth rates of *Stephanodiscus minutulus* with bacteria (hatched bars, A, B) pre-treated with no (C), low (L), and high concentrations (H) of tannic acid (TA) and without bacteria (C, D) but with the sterile filtered culture medium of the bacteria. TA (0.001 mg TA mL^{-1} d^{-1}) was added daily to the respective treatments (grey bars, C_{TA}, L_{TA}, H_{TA}). Different lower case letters in graphs indicate significant differences ($p < 0.05$) between growth rates separately for each treatment (A-D) calculated by one-way ANOVA and subsequent Tukey–B post-hoc tests.

3.5.3 Degradation of allelochemicals by specialized bacterial isolates (phase 3)

All bacteria isolated from bacterial communities pre-adapted to different TA concentrations belonged to Pseudomonads (Gammaproteobacteria). The sequenced isolates B5, B6, B7, B8 and B12 were specified as *Pseudomonas sp.*, whereas isolate B11 is identical to *Pseudomonas putida* (Fig. 26, Table 15 appendix). Isolates B3 (Li et al. unpublished results in NCBI) and

B6 are related to antibiotic resistant strains, isolates B7 and B8 are identical and closely related to pyrene degrading bacteria. Strains B8, B1 (one unidentified bacterium), isolate B11 (related to a quinolene degrading bacterium) and isolate B12 (related to a bacterium involved in sulphur oxidation) were used for TA degradation experiments (*phase 3*). Without addition of TA, concentrations of total phenolic compounds (TPC) of bacteria isolates (B1, B8, B11, and B12) did not significantly differ from the control (ANOVA, $p = 0.291$, $F = 1.44$, Fig. 28). Thus differences in background TPC concentrations should not have influenced the following TA degradation experiment. After addition of TA, bacteria isolates reduced the initial TA concentration (50 mg L^{-1}) by 74 to 82%. Between treatments, we noticed species-specific differences with isolates B11 and B12 being the most effective TA degraders and isolates B1 and B8 being less effective in degrading TA but still reduced TA concentration significantly more than in the control (ANOVA, $p < 0.001$, $F = 42.6$, Fig. 28). Unfortunately, in the respective axenic control we detected some bacteria colonies at the end of the experiment. This may explain the low differences of detected concentration of total phenolic compounds between the control and bacterial isolates.

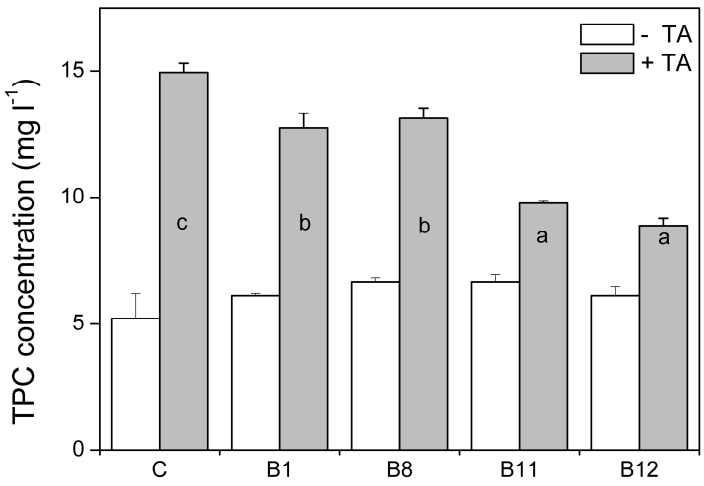


Fig. 28: Concentrations of total phenolic compounds (TPC) of sterile filtered culture medium without degradation (control) and after degradation by bacterial isolates B1, B8, B11, and B12 exposed to 50 mg L^{-1} tannic acid (grey bars, +TA) or not exposed to TA (white bars, -TA). Different lower case letters in graphs indicate significant differences between TPC concentrations after TA degradation by different bacterial isolates and the control (C) ($p < 0.05$) as calculated by one-way ANOVA and subsequent Tukey–B posthoc tests. No significant differences occurred in the treatments without TA exposure (white bars).

3.5.4 Effect of degradation products on *D. armatus*

Growth rates of *D. armatus* exposed to sterile filtered culture solutions including metabolic products/enzymes of the different bacteria isolates without TA did not differ significantly from the axenic control (ANOVA, $p = 0.277$, $F = 1.491$, Fig. 29). This notion indicates that the bacteria culture medium without TA did not *per se* affect growth of the algae under these conditions. The effect of bacterial degradation products of TA was significantly weaker and revealed species-specific differences among isolates (ANOVA $p < 0.001$, $F = 36$, Fig. 29). TA-degradation products always yielded lower growth rates of *D. armatus* compared to sterile filtered culture medium of the respective bacterial isolates but still facilitated growth, whereas TA not exposed to bacterial degradation (C: 50 mg L^{-1} TA, Fig. 29) caused negative growth of *D. armatus*. The degradation products of isolate B8 and B12 reduced algal growth significantly compared to their respective control (with sterile filtered bacterial culture medium) ($p < 0.05$, t-tests, Fig. 29). In contrast, TA degradation products of isolates B 11 and B1 did not lead to a significant reduction in algal growth compared to the respective sterile filtered culture medium of the bacteria. TA degradation products of isolate B8 stopped growth of *D. armatus*. In the presence of degradation products of isolate B11 and B12, which reduced TA concentrations most effectively, growth rate of *D. armatus* accounted for only 33-35% of that of the untreated control (C, Fig. 29).

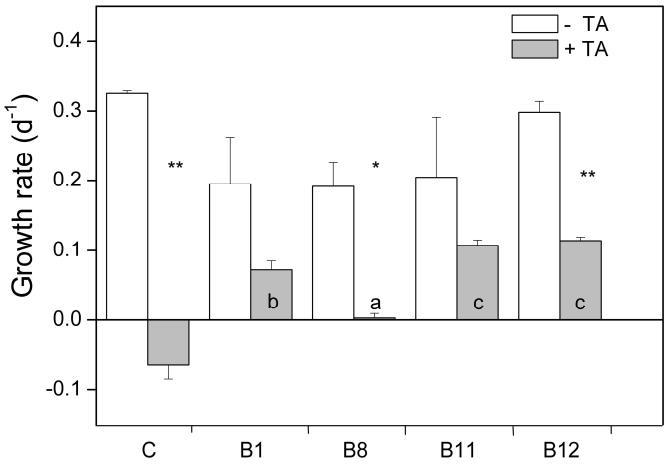


Fig. 29: Growth rates of the axenic green alga *Desmodesmus armatus* without (-TA, white bars) and exposed to degraded tannic acid (TA; +TA, grey bars) by different bacterial isolates (B1, B8, B11, B12) and grown in bacteria free culture medium (C). Different lower case letters in graphs indicate significant differences ($p < 0.05$) between growth rates of *D. armatus* calculated by one-way ANOVA and subsequent Tukey–B posthoc tests (grey bars). No significant differences were found between growth rates in control (-TA; white bars). */** indicates significant differences at $p < 0.05$/$p < 0.01$ between –TA/+TA treatments tested with Student's t-test

3.6 Comparison of sensitivity test *

To evaluate if different experimental approaches to test algal growth response to allelochemicals reveal transferable results we performed coexistence experiments with two green algae *Desmodesmus subspicatus* and *Stigeoclonium helveticum* in dialysis bags exposed to *M. verticillatum* in a lake and in aquaria and with the allelopathic model substance tannic acid in reagent tubes in the laboratory. We further tested for differences of growth parameters based on fluorescence measurements and particle counting.

3.6.1 *In situ* test with test organisms in dialysis tubes

Coexistence experiments performed with dialysis tube in the lake showed a decrease in cell counts and biovolume inside the *M. verticillatum* stands as compared to the control site without macrophytes for both green algae species. This difference, however, was only significan- for the biovolume of *D. subspicatus* (Fig. 30).

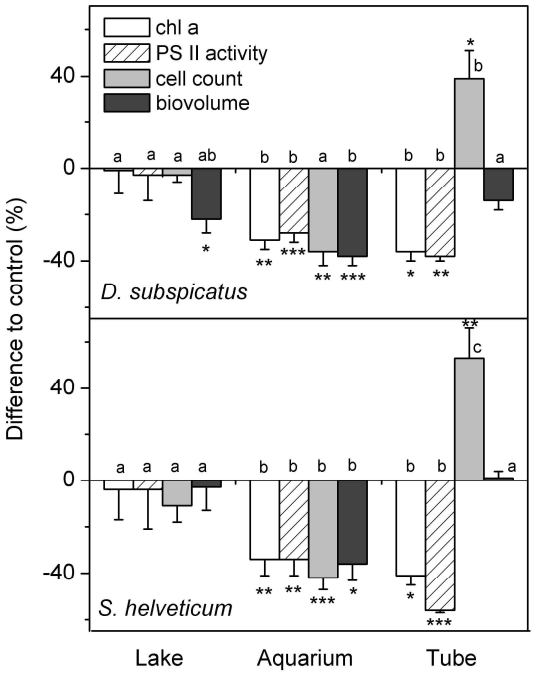


Fig. 30: Difference to control of the chl a concentration, PS II activity, cell count and biovolume of the green algae Desmodesmus subspicatus and Stigeoclonium helveticum grown in dialysis membrane tubes in coexistence with Myriophyllum verticillatum for 48 h in situ in a lake, in an aquarium or in glass tubes after a single addition of tannic acid of 250 µg L-1 (means of four replicates ± standard error; different letters indicate significant differences between methods tested separately for each parameter by one-way ANOVA at $p<0.05$). Asterisks (*, **, ***) indicate significant differences between measured parameters of control and treatment at $p<0.001$, 0.01 and 0.05 (t-tests), respectively. (Hilt, S., Beutler, E., Bauer, N. under revision)

More significant differences may have been possible with a longer exposure time, though this was avoided in order to eliminate the risk of clogged dialysis membranes. A decrease of PS II activity of algae inside the plant stands was expected due to the exudation of polyphenolic allelochemicals by *M. verticillatum* (Hilt et al. 2006) and their known effect on PS II (Körner and Nicklisch 2002). However, chl *a* and PS II activities reduced in both control and macrophyte stands. Growth rates based on chl *a* of both algae species at control sites in the lake were significantly lower than in aquaria and glass tubes (Table 16), indicating generally more adverse growth conditions for algae in the lake (e.g. regarding temperature, light, stirring) as compared to laboratory conditions.

3.6.2 Incubation test with *M. verticillatum* in aquaria

In aquarium experiments all measured growth parameters of the two green algae were significantly lower for algae grown in dialysis membrane tubes among *M. verticillatum* as compared to the control aquaria containing plastic plants (Fig. 30). For this method no significant differences were detected between parameters or algae species (one-way ANOVA, $p > 0.05$).

3.6.3 Laboratory experiment with reagent tubes and TA as test allelochemical

In glass tubes experiments, results were qualitatively comparable to aquaria experiments for fluorescence-based parameters whereas particle-based parameters showed a contrasting pattern with significantly increased cell counts and unchanged biovolume in both algae species (Fig. 30). Mean particle volume was thus significantly reduced by TA treatment whereas it remained unchanged in the lake and aquaria experiments.

3.6.4 Comparison of different approaches of sensitivity tests

At the beginning of all three experiments Chl a concentrations were kept at the same level for each alga species (one-way analysis of variance (ANOVA), $p>0.05$, Table 16). At the end significant inhibitive effects on D. subspicatus as compared to the control were found in the in situ approach only for the biovolume. In aquarium experiments we found significant inhibition of the green algae exposed to shoots of *M. verticillatum* for all measured parameters as compared to control aquaria containing plastic plants (Fig. 30). All parameters and both algae species showed the same % difference between control and treatment with this method (one-

way ANOVA, p>0.05). In glass tubes experiments, inhibitive algal growth response to TA was qualitatively and quantitatively comparable to aquaria experiments for fluorescence-based parameters. Particle-based parameters, however, showed a contrasting pattern with significantly increased cell counts and unchanged biovolume in both algae species (Fig. 30, Table 16).

Table 16: Chl a concentrations, PS II activity (RU: relative units), cell counts and biovolume (means ± standard error) of the green algae Desmodesmus subspicatus (D.s.) and Stigeoclonium helveticum (S.h.) at day 0 before experiments and day 2 in controls (c) and treatments (t) in the lake, the aquarium and glass tubes, and growth rates between day 0 and 2 based on chl a. (Hilt, S., Beutler, E., Bauer, N. under revision).

Parameter	**Time/ treatment**	**Lake**		**Aquarium**		**Tube**	
		D.s.	***S.h.***	***D.s.***	***S.h.***	***D.s.***	***S.h.***
Chl *a* (μgL^{-1})	Day 0	11.1 ± 1.7	6.7 ± 0.4	10.8 ± 0.4	6.9 ± 0.7	8.7 ± 0.5	8.3 ± 0.1
	Day 2 c	35 ± 4	19 ± 1	148 ± 5	89 ± 2	79 ± 9	80 ± 8
	Day 2 t	34 ± 1	18 ± 2	103 ± 6	59 ± 6	51 ± 4	47 ± 4
PS II activity (RU)	Day 0	19.0 ± 0.3	11.3 ± 0.1	18.7 ± 0.9	11.0 ± 0.2	18.0 ± 0.5	15.2 ± 0.5
	Day 2 c	71 ± 8	42 ± 4	295 ± 3	169 ± 4	112 ± 5	138 ± 6
	Day 2 t	67 ± 1	38 ± 4	211 ± 11	112 ± 12	70 ± 3	60 ± 3
Cell count (counts $\bullet mL^{-1}$)	Day 0	7.1±0.3 $\bullet 10^4$	5.1 ±0.1 $\bullet 10^4$	4.1± 0.2 $\bullet 10^4$	5.3±0.4$\bullet 10^4$	7.1±0.03$\bullet 10^4$	6.0±0.08$\bullet 10^4$
	Day 2 c	4.5 ±0.3 $\bullet 10^5$	3.4 ±0.3 $\bullet 10^5$	6.1± 0.3 $\bullet 10^5$	5.8± 0.3 $\bullet 10^5$	2.0±0.1$\bullet 10^5$	2.0±0.07$\bullet 10^5$
	Day 2 t	4.4 ±0.1 $\bullet 10^5$	3.0 ±0.3 $\bullet 10^5$	3.9± 0.4 $\bullet 10^5$	3.4 ±0.3 $\bullet 10^5$	2.7±0.3$\bullet 10^5$	3.1±0.3 $\bullet 10^5$
Biovolume ($mm^3 \bullet mL^{-1}$)	Day 0	4.9 ±0.6 $\bullet 10^6$	5.4 ±1.3 $\bullet 10^6$	2.9 ±0.1 $\bullet 10^6$	2.9±0.3 $\bullet 10^7$	1.2±0.02$\bullet 10^7$	1.0±0.03$\bullet 10^7$
	Day 2 c	5.7 ±0.3 $\bullet 10^7$	3.8 ±0.4 $\bullet 10^7$	4.9 ±0.2 $\bullet 10^7$	1.1±0.1 $\bullet 10^8$	4.6±0.3$\bullet 10^7$	3.7± 0.2 $\bullet 10^7$
	Day 2 t	4.5 ±0.4 $\bullet 10^7$	3.6 ±0.4 $\bullet 10^7$	3.0 ±0.2 $\bullet 10^7$	7.3±1.0 $\bullet 10^7$	3.9±0.1$\bullet 10^7$	3.7± 0.1 $\bullet 10^7$
Growth rates (d^{-1})	c	0.57 ± 0.05	0.53 ± 0.03	1.31 ± 0.02	1.28 ± 0.01	1.10 ± 0.06	1.13 ± 0.05
	t	0.56 ± 0.01	0.49 ± 0.07	1.13 ± 0.03	1.07 ± 0.06	0.88 ± 0.04	0.86 ± 0.05

Overall, the reaction (% difference between control and treatment) of both algae to polyphenolic allelochemicals differed significantly between the three tested methods for all tested parameters (one-way ANOVA, $p < 0.05$). Differences occurred both qualitatively and quantitatively. Fluorescence-based parameters showed a higher similarity between aquaria and glass tube experiments, and particle-based parameters were comparable in lake and aquaria experiments (Fig. 30). Differences in the reaction of the two green algae species to allelochemicals were only significant for the parameter biovolume (two-way ANOVA, $p < 0.05$). The differ-

ence between species was detected independently of the method (two-way ANOVA, species x method, $p>0.05$). However, species specific differences for the reaction of both green algae to allelopathic substances detected by t-tests separately for each method and parameter, showed significant differences only in the glass tube experiment. Here, *S. helveticum* was more sensitive than *D. subspicatus* for PS II activity and less for biovolume. The differential sensitivity of algae to allelochemicals thus cannot be detected independently of the method and parameter applied. Potential reasons for this are species-specific modes of action and interactions with specific bacteria (Bauer et al. 2010, this thesis).

*The presented results of this paragraph are extracted from Hilt, S., Beutler, E., Bauer, N. (Journal of Phycology, under revision)

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4 DISCUSSION

In the last few years, the debate on allelopathy has shifted from doubts on its existence to the importance of allelopathy on ecosystem level (Mulderij 2006; Gross et al. 2007). To evaluate the relevance of allelopathy it is essential to find out which environmental factors influence the outcome of allelopathic effects e.g. for the mechanisms of shifts between the two alternative states in shallow lakes. This work demonstrates the utility of multi-method approaches combining biochemical analytical, molecular biological and physiological investigations in experiments from *in situ* to *in vitro* approaches with increasing complexity to advance our understanding of allelopathy in the aquatic environment.

The objective was to identify factors influencing allelopathic effects of the macrophyte-allelochemical-phytoplankton signal flow path. In the first part of the discussion I will evaluate the results of *in situ* investigations of the temporal dynamics of allelopathic compounds of *Myriophyllum verticillatum* in relation to the phytoplankton response and refer to nutrient content of the plant as a possible factor explaining the observed dynamics of total phenolic compounds in plant tissue. Single phenolic compounds that showed major inhibitive activity in sensitivity tests with Cyanobacteria were identified by liquid chromatography and confirmed the observed temporal dynamics (Paragraph 4.1). In the following I will discuss the effect of abiotic and biotic factors such as microbial and photochemical degradation on the activity of the allelochemical (Paragraph 4.2) or temperature and bacteria on the phytoplankton response to the test allelochemical TA (Paragraph 4.3). The special role of phytoplankton-bacteria interaction is addressed in paragraph 4.4 and 4.5 discussing the effect of different initial phytoplankton concentration, mutualistic relation between phytoplankton and bacteria and the bacterial community composition on the allelopathic effect of TA on phytoplankton species. Finally a discourse of different approaches to detect allelopathic effects of submerged macrophytes on algae based on fluorescence and particle derived parameters is presented to critically question the transferability of results obtained by different approaches (Paragraph 4.6).

4.1 Dynamic of phenolic compounds of *Myriophyllum verticillatum* and phytoplankton response

4.1.1 Intraspecific variation of total phenolic compounds in *M. verticillatum*

In general, submerged macrophytes are assumed to have lower concentrations of phenolic compounds than emergent and floating-leaved aquatic plants due to their lower vulnerability to herbivory, lower exposure to UV-B radiation and lower availability of light (Smolders et al. 2000). These factors (herbivory, light availability and UV exposure) are usually assumed to promote the production of secondary plant metabolites (see 1.2.3 *Introduction*). In the submerged *Myriophyllum verticillatum* we found relative high concentrations of polyphenols that proofed to have allelopathic activity in the sensitivity test with cyanobacteria (Table 6, Fig. 13, 15). The investigated content of total polyphenols of *M. verticillatum* constituted up to 12% of dry mass found in apices and were 2.5 times higher in the closely related model plant *M. spicatum* with 30% of dry mass (Gross et al. 1996). This characterizes the genera *Myriophyllum* as especially allelopathically active among submerged macrophytes due to the specific allelopathic characteristics of their polyphenols (Hilt et al. 2006; Gross et al. 1996). Plant polyphenols (synonym vegetable tannins) can have various functions in plant physiology (see 1.2.1 *Introduction*) including UV protection and plant defence (Hättenschwiler 2000; Hättenschwiler et al. 2003). After release into the environment they may subsequently act as an allelochemical. This may also explain why total phenolic compound (TPC) concentrations found in plant tissue of *M. verticillatum* were highest in apices. We therefore used plant extracts of the apical zone for evaluating the allelopathic effect of *M. verticillatum*. The four years *in situ* sampling campaign revealed a relatively high intra- and interannual dynamic of TPC concentrations in apices of *M. verticillatum* varying by a factor of 4.0 within the whole period of investigation and by a factor of 1.8 to 2.8 within years. Smolders and colleagues (2000) found variations of TPC content in leaves of various submerged macrophytes, which were even higher (up to a factor of 46). However, no reference was made to the depth of the plant stands, the age of the leaves or nutrient conditions in the lakes, all of which might contribute to this higher variability. Variations in our study were lower, probably because we always collected plant material in the same water depth and changes in environmental conditions as e.g. nutrient condition were moderate.

Although there was no consistent seasonal pattern of TPC concentrations in *M. verticillatum* of the Lake Krumme Laake, we found major variability of TPC concentrations in May and

generally higher TPC concentrations in May to July, when the submerged plants grow to the surface. This suggests that allocation of allelopathically active polyphenolic compounds is related to biomass accumulation of the macrophytes. Biomass allocation was reported to be a determinant of tannin concentration in growing plants (Häring et al. 2007). Growth of macrophytes in turn is influenced by changing biotic and abiotic factors such as herbivory (Lodge 1991), epiphytic cover (Sand-Jensen 1990; Gross 2003a) and changes in nutrients (Cronin und Lodge 2003), temperature (Madsen und Brix 1997; Rooney und Kalff 2000) and light climate dependent on water level fluctuations (Blindow 1992). This might explain the relatively high variability during spring and early summer when there are highly fluctuating climate conditions in the northern hemisphere. In the early growing season, when plants have not yet reached the water surface, allelochemical defense may give the macrophytes a competitive advantage over phytoplankton in the struggle for light. The allocation of polyphenolic substances either as a secondary plant metabolites for protection against UV or as allelochemicals both are beneficial for the plant in spring when solar radiation is high and the risk of shading by epiphyton or phytoplankton increases. A more specific view on individual polyphenolic compounds, however, may help to unravel observed pattern of allelopathic content in submerged plants.

4.1.2 Impact of nutrient availability on TPC

In the four investigated years nutrient availability was not the major factor but partly could explain the temporal dynamics of allelochemicals (research question 1). The observed seasonal and interannual variability of total phenolic compound (TPC) concentrations in *M. verticillatum* in Lake Krumme Laake were not strongly and not in all years related to variations in plant tissue nutrient content. Different conceptual models (CNBH: carbon nutrient balance hypothesis: (Bryant et al. 1983); growth differentiation model: (Herms und Mattson 1992); PCM: protein competition model: (Jones und Hartley 1999)) predict higher allocation of phenolics when nutrients are limiting. However, N and P concentrations in *M. verticillatum* apices were most above limiting levels of 13 and 1.3 mg g^{-1} DW. We found weak positive correlation between C/N ratio and TPC in *M. verticillatum* apices for two of four years and in stems and leaves for 2007 when N and P values were not limiting in plant tissue. In *M. spicatum* Gross (2003b) did not find a correlation between TPC and C/N ratio, but did so for tellimagrandin II. She established axenic cultures of *M. spicatum* to avoid interference with the epi-

phytic biofilm, however, axenic plants might react more sensitively than xenic plants and transferability to natural conditions remains difficult. In our study we measured nutrient content of plant tissues. This can only roughly indicate the actual nutrient availability for specific physiological processes, and possible limitations in specific compartments can not be detected by this method. Findings of weak negative correlations between TPC and P in two of four years support the rather scarce information on the impact of P availability on TPC (Hättenschwiler et al. 2003). The production of allelopathic polyphenols that can inhibit the alkaline phosphathase (APA) of phytoplankton may give *M. verticillatum* a competitive advantage if P is limiting. In Lake Krumme Laake, however, N and P are not limiting (Table 2) and thus other factors than nutrient availability are assumed to be more important for the variability in the content of phenolic compounds in plant tissue such as light availability combined with water level fluctuations. This is supported by findings of higher concentration of TPC in the apical tissue of *M. verticillatum* harvested near the water surface compared to apicals harvested in deeper zones (Blaschke 2007). Pavia and colleagues also found a positive correlation between light availability and the content of phlorotannins – secondary plant metabolites that protect the plant against UV in brown seaweeds (Pavia 2000). Detecting light availability for submerged macrophytes *in situ* remains difficult due to interference with phytoplankton and epiphytic organisms that contribute to shading and are subject to seasonal changes. Biotic stressors such as herbivory or other environmental stimuli also influence biochemical plant response and might explain observed TPC variability as was reported for secondary metabolite dynamics of terrestrial plants (Metlen et al. 2009). Finally, trophic interactions such as fish predation on epiphyton-grazing macroinvertebrates may alleviate light limitation for submerged macrophytes (Hrbácek et al. 1961; Sand-Jensen 1991) and thus influence biochemical plant response.

Individual compounds can be differentially regulated by abiotic factors compared to the pool of phenolic compounds. For example, Gross (2000) found spatio-temporal variations in TPC concentrations in *M. spicatum* to be less pronounced than concentrations of tellimagrandin II, its major allelochemical. Results obtained for the major allelopathically active compounds of *M. verticillatum* in 2004 (see 3.1.2 *Results*) show a more distinct seasonal pattern and a clear maximum in May (Fig. 14). This confirms the theory that primary production of allelopathic polyphenols of *M. verticillatum* occurs when the macrophyte grows from the bottom to the water surface together with biomass accumulation at high light availability. Thus, further investigations on the dynamic of allelopathic substances should focus on single allelopathically

active compounds and take into consideration stress by abiotic (light climate) and biotic (e.g. herbivory) stressors and test them separately under controlled conditions on both xenic and axenic plants.

4.1.3 Allelopathically active compounds

The dynamic of total polyphenolic compounds (TPC) in *M. verticillatum* was mirrored by the growth inhibition of the tested cyanobacterium *A. variabilis* (Fig. 15) (research question 1b). The applied agar diffusion assay therefore seems to be an adequate method to identify the most allelopathically active fraction in a plant extract. Cyanobacteria are often used for the detection of allelopathic effects of submerged macrophytes and proved to be a sensitive group as compared to e.g. chlorophytes (Hilt und Gross 2008). Similar tests were used to show the allelopathic activity of *M. spicatum* (Gross et al. 1996), *Najas marina* and *Ceratophyllum demersum* (Gross et al. 2003), *Elodea canadensis* and *E. nuttallii* (Erhard 2006) as well as *Stratiotes aloides* (Mulderij 2007a). Direct comparisons between these tests to rank allelopathic activities of different macrophyte species remain difficult, as culture conditions such as incubation times often differed. Although experiments conducted with extracts cannot give final proof for allelopathic interactions (Gross et al. 2007), they still provide useful information about active compounds and proof if the temporal dynamics of these substances correspond to seasonal and interannual inhibitive activity on phytoplankton (this study).

Bioassay-directed fractionation indicated that only a few compounds contributed significantly to allelopathic activity in *M. verticillatum* extracts. Four phenolic compounds with HPLC-PDA retention times of 22, 24, 25 and 28 min referring to compound numbers 6, 7a, 7b and 8, (Table 8) were mainly responsible for the inhibitory effect. The seasonal pattern of their inhibitory effect on the tested cyanobacterium clearly points to a maximum allelopathic activity of *M. verticillatum* in May and June and shows that phytoplankton response mirrors TPC dynamics in the plant (hypothesis 1). Exudation rates for single compounds were not determined, but TPC exudation rates in former studies on *M. verticillatum* were also highest in May (Hilt et al. 2006). The four identified compounds are isomers of the closely related ellagitannin tellimagrandin II (1,2,3-tri-*O*-galloyl-4,6-*(S)*-hexahydroxydiphenoyl-*ß*-D-glucose = a HHDP-tri-galloylglucose), the major allelopathically active compound in *M. spicatum* (Gross et al., 1996). Final proof of the correct stereochemistry of these compounds is in preparation (Jenett-Siems, Siems, Gross, Bauer & Hilt, unpublished results). HPLC-PDA analysis sug-

gested that these compounds are not identical with the three phenylpropanoid glucosides detected in *Myriophyllum verticillatum* by Aliotta and colleagues (Aliotta et al. 1992).

4.2 Abiotic and biotic influences on the diagenesis of TA and lake DOC and its allelopathic activity on *Desmodesmus armatus*

One of the main critical points that question the allelopathic effect is the loss of the allelopathic activity due to reaction with the aquatic environment and the exposure to physicochemical and microbial degradation processes. Released into the water phenolic allelochemicals may undergo spontaneously chemical reaction such as autooxidation (Appel 1993) or the formation of complexes with proteins and polymerization (Haslam 1998; Spencer et al. 1988) and thereby change its allelopathic activity. Solar radiation can provide substrate by photo oxidation of dissolved organic matter (Granéli et al. 1996) for microbial degradation processes (Lin und Carlson 1991; Wetzel et al. 1995). This may become increasingly important as solar ultraviolet-B radiation will augment due to accelerated depletion of the stratospheric ozone shield (Staehelin et al. 2001).

The results of the second presented study on the influence of solar radiation and bacteria on the allelopathic activity of TA showed remarkable differences in the diagenesis of the allelochemical between sunlight and dark incubated water samples manifested in different DOC size composition of degradion products. The most notably difference found in water samples with the allelochemical exposed to solar radiation was the formation of recalcitrant substances assigned as humic substances (HS) and high molecular weight substances (HMWS), a general decrease of the total phenolic compounds and SUVA, and an accumulation of LMWS (with and without bacteria), (Fig. 17, 18 and 19). In the dark incubated parallel water samples all chromatographically detectable size fractions of DOC decreased and confirmed a rapid turnover of the added TA. We know that individual organic compounds can be incorporated with very different efficiencies (del Giorgio und Cole 1998) and irradiation of DOM can either enhance (Wetzel et al. 1995; Lindell et al. 1995) or reduce bacterial utilization depending on substrate quality (Tranvik und Bertilsson 2001). Photolytic transformation products of phenols such as polymerized coumaric acid were found to constitute a good substrate for heterotrophic bacteria (Tittel 2008). While other studies confirm the accumulation of dissolved organic matter and phenolic compounds resistant to bacterial decomposition in the sunlight in pelagic nsystems (Amon und Benner 1996; Carlson und Mayer 1980). Fresh and labile DOM

can be resistant to UV or rendered more recalcitrant by solar radiation (Keil und Kirchman 1994; Benner und Biddanda 1998). The chromatographically detectable DOC in our experiment was maximal after two weeks of exposure to solar radiation which indicates that regeneration or transformation processes are the prevailing processes responsible for the accumulation of photolytic degradation products like humic substances whereas DOC decreased to minimal values in the dark parallels. Both photolytic degradation by solar radiation and changes in microbial activity due to the antibacterial activity of TA (Scalbert 1991; Walenciak 2002; Buzzini 2008) are assumed to be responsible for this difference in TA diagenesis. Bacterial degradation is further complicated by its high reactivity with carbohydrates, proteins and N-containing compounds promoting oxidation reaction and the formation of complexes and polymerization reactions (Kraus et al. 2004). Reports of reduced bacterial availability of labile DOM after exposure to solar radiation due to limited co-substrates such as low concentrations of humic bound amino acids (Rosenstock et al. 2005) suggest that interactions with the lake water may have occured. It remains, however, obscure which co-substrates in our experiment could have been depleted exclusively in the treatments exposed to solar radiation and not in the dark incubations.

The formation of recalcitrant HS independent of the presence or absence of bacteria suggests that photolytic processes are mainly responsible for this phenomenon. Spontaneous photodegradation of phenols and condensation or polymerization reactions can promote the synthesis of HS (Pajares et al. 2010). These humic substances in turn may affect bacterio- and phytoplankton especially if exposed to sunlight. Irradiated HS are reported to disturb bacterial membrane integrity (Lemke et al. 1995), bacterial enzyme activity (Boavida und Wetzel 1998; Espeland 2001) and bacterial activity in humic lakes via ROS generation observed for polyphenols exposed to solar radiation (Glaeser et al. 2010). The formation of radicals e.g. of semiquinone radicals can be positively (hydrolysable tannins) or negatively (condensed tannins) associated with oxidative activities depending on tannin composition (Barbehenn et al. 2006). ROS in turn can participate in condensation or polymerization reactions and promote the natural synthesis of humic products (Pajares et al. 2010). The formation of ROS or other reactive compounds such as H_2O_2 can induce chain reactions that either accelerate the formation of HS or impede microbial degradation of TA. Natural macrophyte leachates exposed to solar radiation including UV were shown to influence bacterial responses (Judd et al. 2007; Bastidas-Navarro et al. 2009). This is supported by findings of different photo-reactivity of leachates from two freshwater macrophytes (*Phragmites australis* and *Hydrocaris morsus-ranae*) ex-

posed to ultraviolet radiation with lowest bacterial growth efficiencies for highest concentrations of hydrogen peroxide (Farjalla et al. 2001).

In the present experiment the freshly added hydrolysable polyphenol TA can also serve as carbon source for phenol degrading bacteria (Müller et al. 2007; Bauer et al. 2010). The applied TA concentrations were relatively high (50 mg L^{-1}). Minimal inhibitive concentrations of polyphenols on bacteria range between 0.012 and 1 g L^{-1} (Scalbert 1991), thus shifts in bacterial community composition by TA towards resistant bacteria should have occurred. This could be confirmed for bacteria from the same lake exposed to the same TA concentrations with increased bacterial number (see paragraph 3.5.1.2 in Results and 4.5 in Discussion). Thus, bacteria able to withstand TA should have been present at the added TA concentration. Considering the relatively high binding capacity of TA to proteins and other organic material, we assume most of the TA being captured after addition by particulate organic matter reducing its allelopathic activity. Nevertheless, we have to be aware that the pulsed TA addition charged the system with relatively high concentrations of phenolic compounds (30 mg TPC L^{-1}) that are far above in situ excretion of phenolic compounds by *M. verticillatum* in 24 h (2-5 µg TPC L-1;(Hilt 2006)) and thus reflect rather potential than ambient conditions. Compared to our batch culture experiments allelochemicals from macrophytes in situ are exuded continuously and will generate recalcitrant HS as observed in our experiment, which in turn may impede further degradation and thereby manifest the allelopathic impact on the ecosystem.

The allelopathic effect of photolytic and microbial degradation/transformation in sensitivity tests performed with Desmodesmus armatus, grown in lake water samples with TA exposed to solar radiation and bacteria confirmed inhibitive growth response compared to parallels grown in the dark incubated samples with TA. Algal growth was not as strongly inhibited as prior to exposure to solar radiation and bacteria. The allelopathic effect after two and three weeks of photolytic and bacterial degradation was thus lowered but persistent. Nutrient limitation during exposure time was largely excluded by the addition of nutrient rich algal culture medium. Thus exposure to solar radiation and bacteria should be the main factors influencing allelopathic effects on algal growth by either lowering inhibitive effects of TA due to microbial degradation in the dark or resulting in less beneficial degradation/transformation products in the sunlight.

Taken together we could found both qualitative and quantitative changes of the allelochemical TA in lake water after exposure to bacteria and/or solar radiation (research question 2a). Eas-

ily accessible degradation products were readily metabolized by bacteria resulting in reduced chromatographically detectable DOC in the dark, whereas less accessible photolytic transformation products of TA such as recalcitrant HS prevailed in water samples exposed to solar radiation. Humic substances are known to have adverse effects on algal photosynthesis (Steinberg 2006) and exo-enzymes (Kim und Wetzel 1993). Additionally other toxic by-products of TA or free radicals (see above) and the inhibition of beneficial microbial activity e.g. degrading enzyme activity and changes in the bacterial community composition (Piccini et al. 2009) by solar radiation could have aggrevated negative effects on algal growth. We have to keep in mind that we found relative small differences in algal growth in relation to the applied concentration of the TA. Nevertheless, the observed phenomenon demonstrated that phenolic compounds can have persistent allelopathic activity due to the recalcitrant character of their transformation products in the sunlight. Under *in situ* conditions macrophytes continuously release allelochemicals and together with already transformed exudates and other reactive degradation products allelopathic effects on phytoplankton can be manifested. It remains, however, to be shown that these persistent allelopathic effects are strong enough to act under the combined environmental factors *in situ* to sustainably control phytoplankton growth and thereby contribute to the stabilization of the clear water regime in shallow lakes despite photolytic and microbial degradation. A detailed molecular characterization of the photolytic and microbial degradation products and analysis of the formation of reactive substances such as ROS or H_2O_2 is suggested to find mechanisms that explain the observed recalcitrance of conversion products. The observed differences in the diagenesis of allelopathic plant derived polyphenols should be included in further food web and flux modeling differentiating between the photic zone of a lake and dark profundal or day and night.

4.3 Influence of temperature and bacteria on the allelopathic effect

In the future influences of changing abiotic factors such as temperature may be of increasingly importance. Increasing water temperature due to global warming can cause shifts in the seasonal development of phytoplankton (Gerten und Adrian 2000; Scheffer et al. 2001; Winder und Schindler 2004a; Winder und Schindler 2004b; Shatwell et al. 2008). This can lead to mis-match effects due to changes in phytoplankton succession. Algae species may react differently under changed environmental conditions e.g. temperature to allelochemicals

present in the the water and temperature dependent susceptibility of the phytoplankton can be essential for dominance or extinction of phytoplankton.

The tested phytoplankton species in the third study showed temperature dependent different growth response to the test allelochemical TA with qualitative (positive and negative effects) and quantitative differences between species due to the presence or absence of bacteria in the algal culture. Interspecific different sensitivity was found for the two diatoms (*G. parvulum*; *Stephanodiscus minutulus*) being more sensitive to TA than the green alga confirming results of different sensitivity of phytoplankton species to released allelochemicals of *M. verticillatum* under *in situ* conditions (Körner und Nicklisch 2002). Reasons for the different growth responses of phytoplankton species to inhibitors can be attributed to differences in algal resistance mechanisms (Wu et al. 2008), temperature dependent detoxifying enzymes (Perelman et al. 2006) including repair enzymes (Roos und Vincent 1998) and different cell wall structures (see 1.3.2.1 *Introduction*). There is clear information on interspecific differences with respect to the sensitivity of phytoplankton to allelochemicals (Mulderij 2003; Körner und Nicklisch 2002). However, the unexplained phenomenon of intraspecific variability of algal response to TA at different temperatures due to bacterial presence or absence remains to be discussed.

In general, increased bacteria to alga ratio obtained in the presence of TA indicate that bacteria benefited from the addition of the allelochemical. Underlying reasons are decreased algal competition for nutrients (Gross et al. 1996) and increased substrate availability of algal exudates deliberated due to disturbed photosynthesis (Gross 1999; Leu et al. 2002) or defect membrane integrity (Dixon et al. 2004) which subsequently enhances carbon transfer through the microbial loop (Uronen et al. 2007).

Bacteria seem not only to profiteer from the allelopathic effect of TA on phytoplankton but also directly influence algal growth response to TA qualitatively and quantitatively at different temperatures. Dependent on bacterial properties and environmental condition the presence of bacteria can diminish or aggravate the allelopathic effect of TA (see *Introduction* 1.3.2.1 and *Discussion*, next paragraphs). The environmental conditions such as temperature and the dominating phytoplankton species (Grossart 2005; Sapp et al. 2007) determine bacteria composition and thereby may change their mutualistic interaction with phytoplankton. Plant derived substances such as phenolic leachates also change the bacterial community composition (Talpsep et al. 1997). Bacteria capable to mineralize polyphenolic allelochemicals can eventually explain the observed beneficial effect of TA on the growth of *G. parvulum* and *D. arma-*

tus. Positive responses of phytoplankton species to allelochemicals have been found previously (Wang et al. 2009). The observed shift from positive effects of TA and bacteria to negative growth response of *D. armatus* with increasing temperature could indicate a depletion of nutrients or increased competition with bacteria at higher temperatures. We intended to minimize nutrient limitation and competition between bacteria and algae by using low algal start concentration and nutrient rich culture medium. However, at higher temperatures bacterial activity increases (Pomeroy und Deibel 1986) and also the risk of proliferation of bacteria that are specialized to harm algae via lyses or due to parasitic life performance increases and this rather than nutrient limitation could have increased algal sensitivity to allelochemicals.

The absence of inhibitive activity of TA in axenic cultures of *G. parvulum* at 10°C and 15°C indicates that the growth rate of the axenic control was already limited at these temperatures. Benthic algae like *G. parvulum* that live in a biofilm with bacteria are specialized to mutualism. Associated bacteria may provide nutrients to the algae or protect the algae against adverse growth conditions in the open water such as radical species. Tannins as TA are able to form chelates with iron or other metal ions due to the *o*-dihydroxyphenyl groups in their structure (Mila et al. 1996). These Fe (II) complexes are suggested to inhibit the formation of free radicals at Fe oxidation (Andrade et al. 2006). Thus, TA in axenic algal cultures might have replaced bacterial siderophores that can trap Fe.

This study gives first evidence that the susceptibility of phytoplankton to allelochemicals changes with temperature (research question 3). Additionally to the known species-specific differences in phytoplankton sensitivity we found intraspecifity due to the interaction with bacteria. Bacteria present in the phytoplankton cultures substantially changed the quantity and the quality of the allelopathic effects. Proposed mechanisms of bacterial mediation of the allelopathic effect are microbial degradation of the allelochemical and changes in mutualistic interactions with the algae due to changes in bacterial community.

4.4 Influence of different specialized bacteria and initial algal concentration on allelopathic effects on *Desmodesmus armatus*

The investigations of the influence of solar radiation and temperature already indicated that mutualistic interaction of bacteria and phytoplankton are responsible for qualitative changes. Differences in the microbial biofilm of e.g. *Microcystis* strains (Casamatta und Wickstrom

2000) were already suggested to cause differences in their sensitivity to allelochemicals (Mulderij 2005) but have not been investigated until now. In order to find differences of bacterial mediation of the allelopathic effect due to phytoplankton-bacteria interaction algal growth response to the test allelochemical TA were compared cultured either with adapted bacterial communities to algal cultures or unspecific bacteria with parallels without bacteria at different intial algal concentrations. The observed stronger influence of specialized bacteria on the allelopathic effect of TA on algal growth response than that of unspecific bacteria underlined the importance of specific interaction of bacteria and phytoplankton for the allelopathic effect. Results showed qualitative differences (positive and negative) in bacterial mediation of algal growth response depending on initial algal concentration which can be ascribed to changes in the mutualistic interaction of bacteria and the alga.

In experiments with low initial algal concentration bacteria showed a weakening effect on the inhibitory activity of TA on phytoplankton. Here algal derived substrate is limited and the addition of TA may provide additional bacterial substrate. Bacteria can developed mechanisms to overcome inhibitory effects of phenols (Smith et al. 2005) and are able to degrade phenolic leachates (Talpsep et al. 1997) and TA (Bauer et al. 2010) and are suggested to proliferate and dominate the bacterial community while sensitive bacteria disappear. Degradation and / or modification of TA by polyphenol-degrading bacteria strains (Erhan 2002) or inactivation of TA due to the formation of complexes with bacteria derived proteins (Zhao und Zou 2002) and exo-polymers can lower the allelopathic effect of TA on the phytoplankton. Phytoplankton may benefit either from degrading bacterial activity on the allelochemical, passive barrier function of the microbial biofilm that surrounds each algal cell or due reduced negative effects of bacteria on phytoplankton (antagonistic bacteria). The minimal inhibitive concentrations of polyphenols are, however, 10 to 1000 times higher for algae and Cyanobacteria than for bacteria (Gross 1995).

In the experiment with high initial algal concentration bacteria especially specific bacteria adapted to algae cultures aggravated the allelopathic effect. Here abundant algal exudates are the preferable substrate for bacteria compared to the less accessible TA. Decrease of TA by bacteria is lowered or incomplete and not degraded TA can excert its allelopathic activity. In addition toxic by-products of TA may be produced. Furthermore, at high algal densities the intra-specific competition and the risk of infection increase due to higher cell contact. This might have synergistically aggravated the inhibitive effect on the algae. The intra-specific

competition was largely excluded by moderate algal start concentrations and a nutrient rich culture medium in order to keep algae exponentially growing. No significant differences was found for growth rates of untreated controls with specific bacteria between low and high initial algal concentration and microscopic surveilence did not reveal increased lyses of bacteria in the experiment with high initial start concentration. However, we cannot fully exclude competition with bacteria the phycosphere.

Overall, the results of this study confirm a significant influence of bacteria-phytoplankton interaction on allelopathic effects on phytoplankton. Initial algal concentration, bacteria/algae ratio and mutualistic bacteria-phytoplankton interaction influenced the allelopathic effect of TA on phytoplankton (research question 4). The action of specific bacteria can be more important than that of unspecific ones. This clearly points out that the quantitative bacterial presence (bacterial abundance) alone is not sufficient to explain the observed qualitative changes of the allelopathical effect on phytoplankton, hence we have to consider also the bacterial community composition.

4.5 Influence of bacterial community on the allelopathic effect of TA on *Stephanodiscus minutulus*

The bacterial community composition will mainly be determined by the available substrat. Studies on the local adaptation of microbial communities demonstrated that the source and composition of DOM play a significant role for microbial metabolism (Findlay et al. 2003; Hoostal und Bouzat 2008). Metabolization of polyphenolic allelochemicals from *M. spicatum* by bacteria (Müller et al. 2007) and microbiological degradation of quercitrin (Westlake 1963), one of the main allelopathically active compounds found in *M. verticillatum* (Bauer et al. 2009), suggest that specialized bacteria can readily use allelochemicals as substrate and thereby may alter their allelopathic activity. The intention of this study was to show if specialized bacteria adapted to degrade or resist to allelochemicals can diminish the allelopathic effect. Therefore a natural bacterial community was previously exposed to different concentrations of the allelochemical tannic acid (0% TA = untreated natural bacteria, 0.05% TA= low and 0.5% TA = high concentrations of TA). The pre-adapted bacterial communities were subsequently checked for their impact on the growth response to TA of the diatom *Stephanodiscus minutulus*. Isolated single bacteria strains from the obtained bacterial communities were checked for their ability to decrease the phenolic content of TA in culture solution. The result-

ing degradation products were then added to the green algae *Desmodesmus armatus* to find changes in growth response.

4.5.1 Phase 1 (Pre-treatment of the bacteria with TA)

To follow the changes of the bacterial composition after exposure to different TA concentrations and after incubation with *S. minutulus* DGGE banding patterns bacteria were compared. The results confirmed substantial changes of bacterial community composition by TA. Cluster analysis of DGGE banding patterns of attached bacteria revealed three clusters that were dependent on the presence and concentration of TA. Thus, the addition of TA seems to be the main factor shaping BCC in the TA treatments.

For the high TA pre-treatment TA concentrations far above natural concentrations were applied. However, bacteria originated from a natural bacterial community out of a dense stand of polyphenol-exuding macrophytes and were assumed to be resistant to te antibacterial properties of phenolic compounds (Buzzini 2008; Freeman et al. 1990). Inhibitory effects of phenolic compounds are mainly ascribed to interactions with enzymes (Freeman et al. 2001) and more generally to interference with proteins, amino acids, phospholipids and sugars (He 2006). However, specific bacterial communities, e.g. in alpine soils, were shown to be not affected by polyphenols (Baptist et al. 2008) or even benefited from polyphenols by using them as a substrate (Chowdhury 2004; Hempel et al. 2009). These bacteria possess a variety of mechanisms to overcome inhibitory effects of tannins by e.g. modification and degradation of tannins (Scalbert 1991; Chowdhury 2004), dissociation of tannin-substrate complexes, tannin inactivation through high affinity binders, membrane modification and other repair or resistance mechanisms such as metal ion sequestration (Smith et al. 2005). Results show that TA pre-treatment of bacteria resulted in higher DGGE band numbers suggesting a higher diversity of bacteria, supposing that the DGGE banding number are representative for the complexity of the whole bacterial community composition.

TA addition selected for bacterial phylotypes that often occur in humic matter and phenol-rich environments. For example, bacteria closely related to Acidovorax sp., which are widely distributed in humic lakes [Burkert, 03; Newton, 06; Grossart, 08]. Other sequenced bacterial strain were representatives of the Betaproteobacteria which belong to the Burkholderiales of

the Comamonadaceae group (Limnobacter sp.) and are common in lakes with high humic matter content (Hutalle-Schmelzer 2010).

4.5.2 Phase 2 (*Stephanodiscus minutulus* cocultured with pretreated bacteria)

4.5.2.1 *Effects of TA and Stephanodiscus minutulus on BCC*

Specific interactions between bacteria and phytoplankton indicate that bacteria have the potential to interfere with allelochemical effects on phytoplankton, e.g. via competition for nutrients (Grossart 1999) removal of cyanotoxins in biofilms (Babica 2005) or mineralization processes within algal aggregates (Grossart 2005; Grossart 2003). Here, the high applied TA concentrations did not finally reduce bacterial growth of the whole bacterial community. New bacterial strains emerging after exposure to high TA in co-culture with *S. minutulus* suggest that these bacteria might be able to resist and/or degrade TA or indirectly benefit from harmed algae by TA. With the applied method (DGGE) for the analysis of the different bacterial communities only relative judgments in comparison to the parallel investigated bacterial communities are possible, because DGGE is not sensitive enough to detect all present bacterial species. In general, the sequenced selection of bacteria rather documented a shift of bacterial community composition towards specialized bacteria resistant to TA or able to use TA as substrate. This could be confirmed by sequencing of bacteria that emerged after exposure of bacterial community to TA. Sequence new emerging bacteria were closely relatives to phenol degrading bacteria such as *Delftia acidovorans*, a Betaproteobacteria that belongs to the Burkholderiales (Comomonadacea) in the natural bacterial community treated with TA in the co-culture experiment with *S. minutulus* in *phase 2*. *Delftia acidovorans* is capable to grow on phenolic compounds (congeners of linear alkylbenzensulfonate such as sulphophenylcarboxylates (Schleheck et al. 2004). Another specialized bacterium, *Rhodoferrax ferrireducens*, was found after TA exposure but also in the untreated bacterial community sampled in the natural macrophyte stand. This confirms that the used bacterial community was composed of already specialized bacteria. In the treatment that was closest to natural conditions (bacterial community pre-treated with low TA concentration and cultured together with the diatom during *phase 2* with daily addition of TA), we found *Erythrobacter*, a specific Alphaproteobacteria, whose marine relatives possess epoxide hydrolase (Woo et al. 2007). This enzyme is involved in detoxification of phytochemicals and allelochemicals by metabolizing plant epoxides and is widely distributed in herbivorous arthropods (Mullin 2009).

Phytoplankton also affected bacterial community composition in phase 2 of the experiment. This could be deduced from increased number of DGGE bandings indicating emerging bacteria in the presence of *S. minutulus* (with and without TA). Phytoplankton-bacteria interaction may have changed in the TA treatment. Average bacterial growth was lowered in culture together with the diatom (*phase 2*) compared to parallels in the absence of the diatom. This observation implicates that we have to consider both direct and indirect effects of TA and its degradation products on either bacteria or algae and on the interaction between algae and bacteria. The phytoplankton provides algal derived dissolved organic matter (DOM) by the release of e.g. mucus polysaccharides or cell components due to lyses by TA, which may interact with TA and can be utilized by the bacteria. The reduction of bacterial growth in the presence of a diatom could be due to increased competition or changes in DOM quality. Other factors such as virus infections, grazing, and nutrient limitation as well as antibiotic activity could also explain such decoupling effects (Abreu et al. 2003), however, were largely excluded by inoculation of axenic well growing algae with filtered bacterioplankton.

TA addition favors phenol degrading bacteria which lead to an accumulation of degradation products and / or complete mineralization of the polyphenol. Subsequent bacterial use of TA degradation products (derived from primary phenol degraders) and released nutrients (due to TA-induced mortality of organisms) in turn can enhance growth efficiencies of other members of the microbial community (De Rito et al. 2005). Hence, emergence of specific bacteria following the TA addition can be addressed to substrate specific growth on either TA degradation products or algal derived cell compounds.

4.5.2.2 Bacteria-mediated effects on algal sensitivity to TA

Daily addition of TA (1µg TA L-1) to cultures of Stephanodiscus minutulus growing in filtrates of the bacterial communities adapted to the different TA concentration resulted in concentration dependent algal growth response (taking into account the background concentration of TA from the pre-treatment). If TA was not further added, the bacteria (adapted to 0% and 0.05% TA) were able to convert the negative allelopathic effect into a stimulation of algal growth probably by the mineralization of TA (Smith et al. 2005). However, bacteria adapted to high TA concentration could not diminish the inhibited algal growth by further addition of TA due to the high applied TA concentration in the pre-treatment. Nevertheless, these results confirm the increasing weakening impact of specialized bacteria adapted to TA. The allelo-

pathically active TA still affected algal growth at daily addition in the presence of specialized bacteria. Repeated addition of TA may cause irreparable harm to bacteria, e.g. membrane damage (Funatogawa 2004; Campos et al. 2009) and decrease diversity of bacteria and thereby lowered the degrading capacity of the remaining bacterial community (Loh und Wang 1998). In addition, incomplete or limited degradation of TA due to reduced bacterial diversity might result in highly toxic degradation products. Similar effects can be expected, when the allelochemicals are continuously released in dense stands of macrophytes. This might explain why allelopathic inhibition of algae can often be detected solely under continuous influence of allelochemicals and not after single TA additions (Hilt et al. 2006)

4.5.3 Phase 3 (Isolated bacteria degrading TA and effect of degradation products on *Desmodesmus armatus*)

Isolated bacteria capable to use the TA as sole carbon source could be identified as Pseudomonads. Pseudomonads are known to degrade aromatic compounds such as polyphenols (Watanabe et al. 1998; Heinaru 2000) or are indirectly involved in phenol degradation (De Rito et al. 2005). Relatives of Pseudomonas have been isolated from antibiotic waste water (Li et al., unpublished results in NCBI). However, our intention was not to select for antibiotic-resistant strains or to confirm the antibiotic activity of tannin. These strains were excluded from the TA degradation experiments. Instead, isolates that are closely related to bacteria which can degrade aromatic compounds (e.g. quinoline, Xu et al., submitted 2009 in NCBI database) or performed sulphate oxidation (unpublished results in NCBI database), were used for degradation experiments (Table 15, Appendix).

TA concentrations decreased after incubation with all tested bacterial isolates, which indicates that the isolated bacteria were capable to degrade aromatic compounds. Besides microbial degradation of TA, photochemical effects (Ervin und Wetzel 2003) cannot be excluded but should have been the same in all treatments and in the control. The axenic control always showed a significantly higher total phenolic content than the samples with bacterial degradation of isolated bacterial strains. Degradation products were then tested for growth effects on Desmodesmus armatus.

The observed growth responses of *Desmodesmus armatus* coincided with the degrading activity of the tested isolated bacterial strains. Changes of allelopathic effects on phytoplankton by

specialized bacteria adapted to the allelochemical can be attributed to bacterial degradation processes besides chemical and physical ones.

Taken together this study demonstrates for the first time that differences in phytoplankton sensitivity (*S. minutulus*) to TA can be assigned to differences in accompanying bacterial community composition (research question 5). Different pre-adapted bacterial communities proofed to be able to differentially change phytoplankton growth response to TA. Additionally, it could be demonstrated that different modes of TA addition resulted in distinct growth responses of the tested alga: a) growth stimulation of axenic algae due to additional substrate and energy supply from previously mineralization of TA by specialized bacteria and b) concentration-dependent inhibition of algal growth by repeated minimal TA concentration even in the presence of specialized bacteria. Taken together the effect of bacterial composition on the phytoplankton and the allelochemical and the mode of addition of the allelochemical might be more important for the resulting allelopathic effect than the absolute concentration of allelochemicals that generally occur in low concentrations.

4.6 Comparison of different approaches for sensitivity tests

Algal growth assays using unicellular liquid cultures of green algae in the laboratory are well established bioassays to investigate phytotoxic effects of chemicals (Lewis 1990). For ecological research questions, however, growth conditions that resemble natural conditions are preferred such as for example in situ experiments under environmental conditions. Under field conditions interference with other factors and nutrient competition cannot be separated from allelopathic effects - if it is at all possible (Inderjit und Del Moral 1997). Consequently, most sensitivity test use plant extracts or purified allelochemicals in laboratory experiments and coexistence experiments. Comparison of results from different approaches can be complicated by different methodological procedures or different measured growth parameters to detect algal sensitivity. This questions the transferability of results of different approaches.

The compare of the results obtained from in situ, aquaria and reagent tube experiments demonstrate that the qualitative algal response (inhibition or enhancement) was the same for the tested algae for growth parameter based on fluorescence, however, for particle detection the sensitivity of algae to allelochemicals cannot be detected independently of the method applied (research question 4). Differences between applied methods may result from time dependency

of growth parameter or different sensitivity of the alga due to sub-optimal growth conditions. In the performed in situ experiment with lowest algal sensitivity (significant inhibition only for biovolume) the growth rates of the two green algae were considerably lower than in the aquaria and reagent tube experiments indicating limited growth conditions probably due to the effect of humic substances in the lake water (see 4.2 this section). Thus growth response after 48h will have underestimated the real allelopathic effect on phytoplankton under in situ conditions and explain the lower sensitivity - longer exposure, however, could have clogged the membranes of the reagent tubes.

Best resolution for species specific differences in the sensitivity of phytoplankton revealed the reagent tube approach. It was very sensitive and turned out to be the only bioassay by which species specific differences between the green algae were detected. This can be explained by optimal growth conditions (light, nutrient and stirring). However, single addition of relative high TA concentrations to the algae as compared to the continuous supply of allelochemicals in low concentrations in the lake and aquaria experiments may have disturbed algae-bacteria interaction or algal cell-cycle dynamics. Deviating results of particle based parameter in the reagent tube experiment show that these tests run the risk of unrepresentative reactions of the algae depending on the parameter and the tested phytoplankton species. The specific property of specific chlorophytes of multiple propagation of the mother cell within each cell cycle (Altenburger et al. 2008) could be a possible explanation for the observed difference in cell division growth and biovolume dynamics in the test tube experiments. Altenburger and colleagues (2008) did observe similar confusing effects on growth with Desmodesmus subspicatus when using particle-based parameters. They suggested an increased frequency of cell divisions of premature cells resulting in small cells after the disturbance of cell-cycles by toxins. This effect has been also observed for the green alga *Scenedesmus quadricauda* after application of lethal toxicant concentrations (Prokhotskaya et al. 2003).

The mode of addition (single addition compared to continuous release in situ and aquaria approach) is assumed to be the main reason for deviating results between particle based and fluorescence based parameters detected in the reagent tube bioassays. This could have been avoided by semi-continuous addition of small concentration of the tested allelochemical. This was considered in all sensitivity tests of phytoplankton to TA of the other studies of this work (experiments 1-5). At high concentrations pulsed polyphenol addition may have changed the present bacterial community due to its antibacterial properties (Scalbert 1991; Buzzini 2008).

Continuous addition of low allelochemical concentrations, however, can select for resistant polyphenol degrading bacteria (see 4.5 of Discussion), which specifically may influence either the properties of the allelochemical or the susceptibility of the phytoplankton. In order to separate the bacterial effect from the effect of the allelochemical parallel test with axenic algal cultures are the only alternative.

Overall, fluorescence-based measurements revealed more reliable results comparing algal growth response to allelochemicals between the different approaches than particle based parameters as cell counts. Recommendation of multiple approaches of different complexity (Gross et al. 2007) and the detection of several growth parameters is strongly supported by these results. It is also recommended to include the detection of algal responses to other modes of action of the allelochemical, for instance, enzyme inhibition or induced ROS formation. The use of dialysis tubes in aquaria seems to be a compromise between the very sensitive reagent tube bioassays and the less sensitive in situ dialysis bag experiments. This method should allow a proper qualitative detection of the reaction of algae exposed to continuously released allelochemicals when using short incubation times and sufficient nutrient supply. However allelopathic effects that are only detectable in nutrient-deficient algae such as the inhibition of the alkaline phosphatase activity by polyphenols (Gross et al., 1996) cannot unequivocally be shown by coexistence experiments (Hilt et al., 2006).

5 CONCLUSION

The objection of this study was to investigate temporal dynamics of allelopathically active substances from *Myriophyllum verticillatum*. Moreover the identification of factors that influence the allelopathic effect on the phytoplankton should be used to complement our knowledge on allelopathy and explain the observed variable performance of allelopathic effects.

The presented four years study of the temporal dynamic of allelochemicals of *Myriophyllum verticillatum* are the first investigations on the temporal dynamics of macrophytes on a longer time period. A detailed analysis of the main allelopathically active compounds revealed patterns of maximal allocation for M. verticillatum during the active growth of the submerged macrophyte from the buttom of the lake to the surface. This is a critical period in the development of macrophytes to overcome competition with planktonic and epiphytic organisms for light and a starting point for phytoplankton succession after the break down of dominating diatoms in late spring of most lakes in Northern Europe. Predicted future increasing temperature and changes in terrestrial run offs with changes in DOC, productivity and biogeochemical cycling increase eutrophication (Pastor et al., 2003) and underlines the importance to understand the mechanisms of regulation of phytoplankton growth by allelopathy. However, further long time studies are necessary to verify if these patterns represent a general performance among allelopathic active macrophytes. The high observed variability of seasonal and interannual allocation of allelopathically active polyphenols demonstrates a high plasticity towards abiotic and biotic factors with nutrient and light availability among others. Therefore we can expect predicted future warming with fluctuations in water level of shallow lakes to have consequences for allocation of allelochemicals in macrophytes.

This risk of changes in allelopathic interactions and mutualistic interaction between bacterio- and phytoplankton due to fluctuating environmental conditions was also demonstrated by temperature dependent algal growth response to allelochemicals and microbial mediation of the allelopathic effect dependent on community composition. Species specific changes of allelopathic effects from positive to negative growth response of phytoplankton will have consequences for phytoplankton composition and trophic interactions.

The use of multi-method approaches and experiments of different complexity to investigate the effect of the combined influence of e.g. bacteria and solar radiation under close to in situ conditions provided evidence that plant derived polyphenolic compounds have the potential to cause persistent inhibitory effects despite transformation and degradation processes in natural

lake water. Qualitatively and quantitatively changes of the allelochemical dependent on prevailing microbially or photochemically degradation processes can manifest or weaken the allelopathic effect on phytoplankton according to prevailing environmental conditions. This amplifies our knowledge of allelopathic substances of macrophytes contributing to the stabilize the clear water state of shallow lakes and should be included in further food web and flux modeling differentiating between the photic zone of a lake and dark profundal or day and night. Nevertheless, results of restricted transferability of different approaches underline the importance of verification of the obtained results under *in situ* conditions

Taken together the high temporal dynamic of allocation of allelopathic compounds in *Myriophyllum verticillatum* and the variable influence of abiotic and biotic factors on the allelopathic effect and the activity of allelochemicals demonstrate the high plasticity of allelopathic action to respond to environmental changes in the aquatic ecosystem. The identified influenced factors by multi-approach experiments add to our knowledge of understanding of allelopathy and explained the observed variable allelopathic effects on phytoplankton.

6 SYNOPSIS

Allelopathy in aquatic ecosystems is a complex mechanism between the donor and target organisms and cannot be regarded separately from abiotic and biotic conditions of the ecosystem. The identified abiotic and biotic factors act on each of the three steps of the allelopathic reaction pathway donor organisms - allelochemical - target organism and were 1) the temporal dynamics of allocation of allelochemicals in the donor plant, 2) the influence of bacteria and solar radiation on the allelopathic activity of allelochemicals and 3) biotic (bacteria) and abiotic (temperature) factors influencing the allelopathic effect on the target organisms. A summary of the reported processes interfering with the allelopathic action of macrophytes on phytoplankton is presented in Fig. 31.

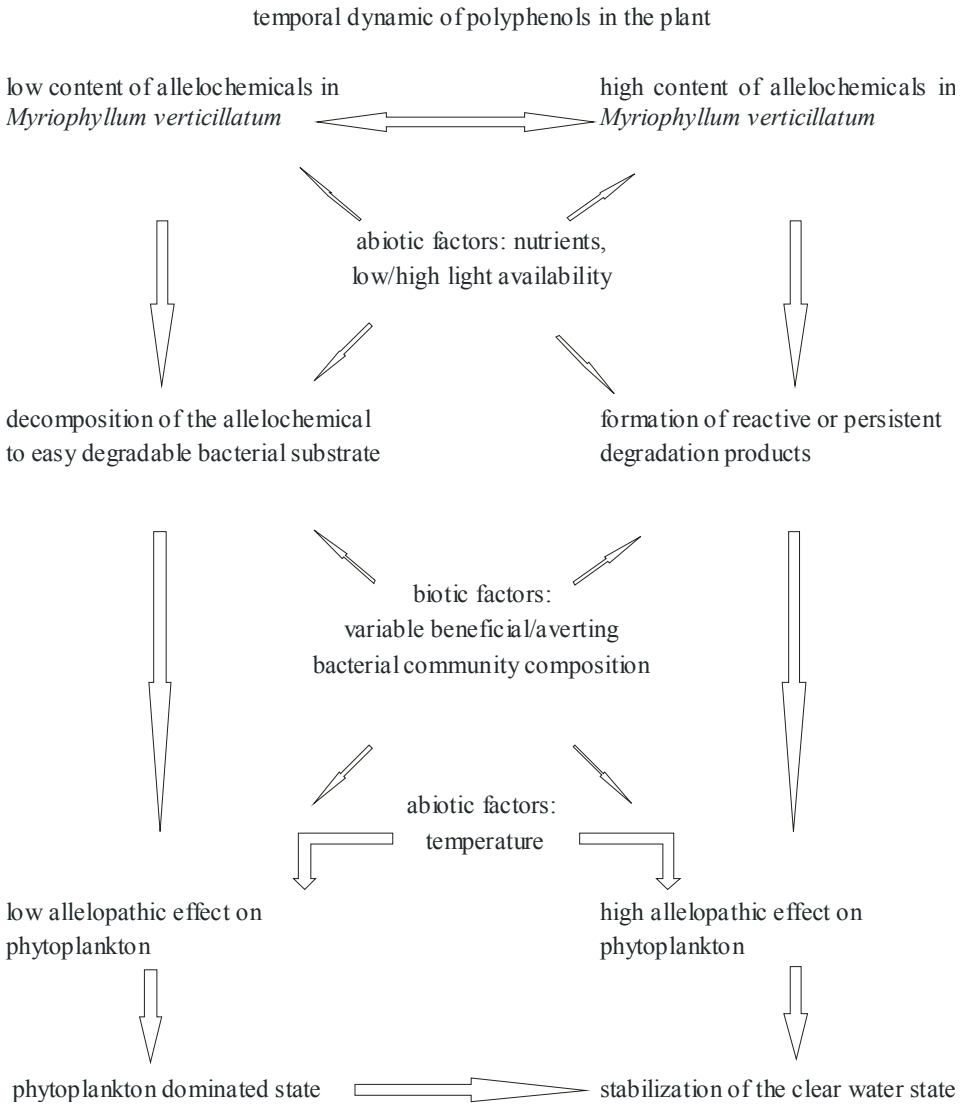

Fig. 31: Influence of investigated abiotic and biotic factors on the allelopathic effect of macrophytes on phytoplankton with effects on the alternating states of bistability of shallow lakes.

First, quantitative and qualitative changes of the allelopathic compounds can determine the allelopathic effect on the phytoplankton. The temporal dynamic of allelopathically active polyphenolic compounds of *Myriophyllum verticillatum* prove a highly variable seasonal and interannual pattern (based on four years of investigation) of the content of allelochemicals under *in situ* conditions. Differences of allelopathically active polyphenolic compounds were up to one order of magnitude both within seasons and among years. Nutrient availability alone was not the dominant factor to explain the variability of polyphenol content in *M. verticillatum* and other factors such as light availability, water level or herbivory might be more important and should be included in future studies. The high variability between the investigated years impeded a detection of a general pattern, however, a detailed view on individual phenolic compounds showed maximal concentrations of the main allelopathically active po-

lyphenols in May. During this time competition between macrophytes reaching the water surface and phytoplankton for light is high and may provide a competitive advantage. The bioassay supported analysis of the allelopathically active compounds from *M. verticillatum* identified isomers of Hexahydroxydiphenoyl -di- and -tri-galloylglucose by liquid chromatigraphically analysis coupled with MS. This compound is close related to the main allelopathic active substance tellimagrandin II in *M. spicatum* and structurally similar to tannic acid, a very common plant polyphenol.

Secondly qualitative and quantitative changes by abiotic and biotic factors after release into the aquaeous medium can alter the reactivity of the allelochemical and thereby the allelopathic effect on the phytoplankton. Bacterial degradation and photolytic processes are among the most important factors influencing the fate of allelochemicals and were investigated using the test allelochemical tannic acid. In contrary to our expectations, combined bacterial and photolytic conversion processes did not synergistically decrease the allelopathic effect of the investigated polyphenol. Physicochemical transformation exposed to solar radiation resulted in recalcitrant degradation products such as humic substances with subsequent long lasting inhibitive effects on the growth of algae compared to degraded polyphenols in the dark. The manifested allelopathic effect of recalcitrant TA degradation products formed by solar radiation together with freshly released allelochemicals favors the hypothesis of macrophytes contributing to the stabilization of the clear water state of shallow lakes. Integrating allelopathy in food web and flux modeling requires a more differentiated view on allelopathic processes, taking into account different diagenesis of allelochemicals in the photic zone of a lake and in the dark profundal and during day and night.

Third abiotic and biotic influences on the target organisms underlined the influence of bacteria for the outcome of the allelopathic effect dependent on temperature and species-specific interaction of phytoplankton and bacteria. Different initial algal concentrations resulting in different mutualistic bacteria-phytoplankton interactions and distinct specialized bacterial communities due to adaption to either the phytoplankton species or to the allelochemical proved to be relevant for the allelopathic effect on the phytoplankton. Polyphenol degrading bacteria selected by pre-treatment to TA diminished the inhibitive allelopathic effect while specific bacteria adapted to the phytoplankton aggravated the allelopathic effects. Thus the composition of the bacterial community is important for the quality and quantity of the resulting effect on the phytoplankton in the investigated system.

Comparing different approaches (*in situ-,* aquaria- and reagent tube experiments) to test allelopathic effects on the growth of two green algae revealed qualitatively transferable results for fluorescence based algal growth parameters while particle based growth parameters deviated for the reagent tube experiment probably due to pulsed addition of the allelochemical. Species specific differences could be found for reagent tube experiments. Thus results from different approaches using different growth parameters have to be interpreted with caution. The application of multi-approaches with increasing degree of realism e.g. from laboratory to *in situ* experiments and multi-methods approaches detecting several parameters or different modes of allelopathic action are recommended to avoid misinterpretation.

Based on multi-method approaches the obtained results confirm that a more differentiated view on allelopathic action including influencing factors for evaluating allelopathic effects of macrophytes on phytoplankton is necessary to evaluate the relevance of allelopathic effects in aquatic ecosystems. We have to expand the unidirectional view of allelopathic effects of macrophytes on phytoplankton to a more complex biochemical interface between macrophytes, phytoplankton and bacteria as well as biochemical and physical factors in the aqueous ecosystem. Results of this study obtained under conditions from laboratory scale to close to natural ones unambiguously demonstrated that transformation products of allelochemical can have long lasting allelopathic effects on phytoplankton and thereby might be important for the stabilizing potential of macrophytes on the clear water state of shallow lakes. However, it remains to be shown if the combination of abiotic and biotic influencing factors and the transformation products indeed can contribute to the clear water state of shallow lakes under *in situ* conditions.

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Publications and presentations

Published publications

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Bauer, N., Hilt, S., Grossart, H.-P. (2009) Effects of bacterial communities on the sensitivity of Phytoplankton to tannic acid." 3. Congress of European Microbiologists (FEMS), poster, Götheburg, Schweden

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Eidestattliche Erklärung

Hiermit erkläre ich, dass ich diese Arbeit eigenständig verfasst habe und bestätige den Wahrheitsgehalt mit meiner Unterschrift.

Ich habe mich anderwärtig nicht um einen Doktorgrad beworben und besitze einen entsprechenden Doktorgrad nicht. Die dem Verfahren zu Grunde liegenden Promotionsordnung der Mathemathisch-Naturwissenschaftlichen Fakultät I der Humboldt Universität zu Berlin habe ich zur Kenntnis genommen.

Berlin, den 12.05. 2011

List of frequently used Abbreviations/ Abkürzungsverzeichnis

ANOVA	Analysis of variance
ADA	Algal diffusions assay
BCC	Bacterial community composition
Chl *a*	Chlorophyll *a*
C/N	Carbon to Nitrogen ratio
DAPI	4′,6-Diamidin-2-phenylindol
DGGE	Denaturing Gel Gradient Electrophoresis
DICE	Correlation index of cluster analysis
DOM	Dissolved organic matter
DOC	Dissolved organic carbon
DW	Dry weight
EDTA	Ethylenediaminetetracetid acid
F_0	minimal fluorescence
Fm	maximal fluorescence
Fv/Fm	quotient of variable fluorescence and maximale fluorescence
HPLC	High performance liquid chromatography
HHDP	Hexahydroxydiphenoyl
HMWS	High molecular weight substances
HS	Humic substances
LCMS	Liquid chromatography mass spectrometry
LC-OCD	Liquid-Chromatography-Organic-Carbon-Detection
LMWS	Low molecular weight substances
OD	Optical density
PhAR	Photosynthetic active radiation
PS	Photosystem
PVP	Polypyrrolidone
ROS	Reactive oxygen species
RP-HPLC	Reverse phase high performance liquid chromatography
SE	Standard error
SPE	Solid phase extraction
TA	Tannic acid
TN	Total Nitrogen
TP	Total Phosphorus

TPC	Total phenolic content
UPGMA	Correlation index of cluster analysis

Appendix:

Table 15. Phylogenetic affiliation of sequenced DGGE bands (indicated with "DGGE" in community label and accession number) of attached (att.) and free living (f.l.) bacteria with no (C), low (L) and high (H) TA treatment of *phase* 1 (*phase* 1) and with and without further TA addition of *phase* 2 (*phase* 2, C_{TA},L_{TA},H_{TA} or C, L, H) and isolated bacteria of *phase* 3 (*phase* 3, indicated with "Iso" in the community label) to closest relative in NCBI gene bank. For phylogenetic relationship of representatives of DGGE and isolates see phylogenetic tree based on 16S rDNA (Fig. 26).

DGGE / Iso bands with community labels & accession No.	**att. / f.l: bacteria**	**TA-treatment** C (0% TA) L (0.05% TA) H (0.5% TA)	**Phylum or group**	**Family**	**Next cultivated relative**	**Accession No.**	**Similarity [%]**	**Closest relative in the Gene bank**	**Accession No.**	**Max. ident [%]**
KLnbDGGE_1 GQ332384	**att.**	C (phase 1)	Proteobacteria; Betaproteobacteria; Burkholderiales	Comamonadaceae; Acidovorax.	*Acidovorax avenae subsp. citrulli* AAC00-1, complete genome	NC 008752	97	Uncultured bacterium clone 655935 16S ribosomal RNA gene, partial sequence	DQ404721.1	**97**
DGGE 3		L (phase 1)	Proteobacteria; Alphaproteobacteria; Rickettsiales	Rickettsiaceae; Rickettsieae; Rickettsia; typhus group	*Rickettsia canadensis str.* McKiel, complete genome	AACY020000000	86	Uncultured bacterium clone 1P-2-L11 16S ribosomal RNA gene, partial sequence	FJ562174.1	**89**
DGGE 4		L (phase 1)	Proteobacteria; Betaproteobacteria; Burkholderiales	Burkholderiaceae; Limnobacter	*Limnobacter sp.* MED105 1103186003328, whole genome	NZ ABCT01000008.1|	96	*Limnobacter sp.* Cy2 16S ribosomal RNA gene, partial sequence.	FJ377408.1	**96**

					shotgun sequence.					
KLnbDGGE 5 GQ332385		L (phase 1)	Proteobacteria; Alphaproteobacteria; Rhodobacterales	Rhodobacteraceae; Rhodobacter	*Rhodobacter sphaeroides* ATCC 17025 plasmid pRSPA02, complete sequence	ATCC 17025	95	Uncultured Rhodobacteraceae bacterium clone Gap-2-91 16S ribosomal	EU642175.1	**98**
KLnbDGGE 6 GQ332386		H (phase 1)	Actinobacteria; Actinobacteridae; Actinomycetales	Streptomycineae; Streptomycetaceae; Streptomyces	*Streptomyces avermitilis* MA-4680, complete genome	NC 003155.4	91	Uncultured bacterium partial 16S rRNA gene, clone L50Sp-17.	AJ966223.1	**99**
KLnbDGGE 7 GQ332393		C (phase 2)	Proteobacteria; Betaproteobacteria; Burkholderiales	Comamonadaceae; Rhodoferax.	*Rhodoferax ferrireducens* T118, complete genome	NC 007908.1	96	Uncultured Comamonadaceae bacterium clone LW18m-1-58 16S rRNA gene, partial sequence.	EU642288.1	**98**
DGGE 8		C_{TA} (phase 2)	Proteobacteria; Betaproteobacteria; Burkholderiales	Comamonadaceae; Delftia	*Delftia acidovorans* SPH-1, complete genome	NC 010002	94	Comamonadaceae bacterium IK1_39 gene for 16S rRNA, partial sequence, strain: IK1_39	AB461020.1	**92**
KLnbDGGE 10		L_{TA} (phase 2)	Proteobacteria; Alphaproteobac-	Erythrobacteraceae;	*Erythrobacter litoralis*	NZ AAMW010	88	Uncultured bacterium partial 16S	FM201099.1	**91**

			teria; Sphingomonadales	Erythrobacter	HTCC2594, complete genome	00002.1		rRNA gene, clone MBR-8_LF_BF90.		
KLnbDGGE 13 GQ332395		H_{TA} (phase 2)	Actinobacteria; Actinobacteridae; Actinomycetales	Streptomycineae; Streptomycetaceae; Streptomyces	*Streptomyces avermitilis* MA-4680, complete genome.	NC 003155.1	91	Uncultured bacterium partial 16S rRNA gene, clone L50Sp-17.	AJ966223	**99**
DGGE 14		H_{TA} (phase 2)	Proteobacteria; Betaproteobacteria; Burkholderiales	Comamonadaceae; Rhodoferax	*Rhodoferrax ferrireducens* T118, complete genome	NC 007908.1	83	Uncultured Comamonadaceae bacterium clone GC1m-2-16 16S ribosomal RNA gene, partial sequence	EU640750.1	**85**
KLnbDGGE 15 GQ332396	**f. l.**	C (phase 1)	Proteobacteria; Gammaproteobacteria; Legionellales	Legionellaceae; Legionella	*Legionella pneumophila str. Paris,* complete genome	NC 006368.1	95	*Legionella pneumophila* isolate DGGE band 4 16S ribosomal RNA gene, partial sequence	DQ408661.1	**95**
KLnbDGGE 16 GQ332397		C (phase 1)	Proteobacteria; Gammaproteobacteria; Legionellales	Legionellaceae; Legionella.	*Legionella pneumophila str. Corby,* complete genome	NC 009494.1	90	*Legionella beliardensis* strain Montbeliard A1 16S ribosomal RNA gene, partial sequence	AF122884.2	**91**

KLnbIso3 GQ332384		Proteobacteria; Gammaproteo-bacteria; Pseu-domonadales	Pseudomo-nadaceae; Pseudomo-nas	*Pseudomonas libanensis strain a105 partial sequence.*	EU434380	99	*Pseudomonas libanensis* strain a105 16S ribosomal RNA gene, partial sequence	EU434380	**99**
KLnbIso5 GQ332385		Proteobacteria; Gammaproteo-bacteria; Pseu-domonadales	Pseudomo-nadaceae; Pseudomo-nas	*Pseudomonas sp.* 104 16S ribosomal RNA gene, partial sequence.	FJ013262	99	Uncultured bacte-rium clone nbt27f08 16S ribosomal RNA gene, partial se-quence	EU535591	**100**
KLnbIso6 **GQ332386**		Proteobacteria; Gammaproteo-bacteria; Pseu-domonadales	Pseudomo-nadaceae; Pseudomo-nas	*Pseudomonas sp.* 104 16S ribosomal RNA gene, partial sequence.	FJ013262 GI:219551897	99	*Pseudomonas sp.* 104 16S ribosomal RNA gene, partial sequence.	FJ013262 GI:219551897	**99**
KLnbIso7 **GQ332387, KLnbIso8** **GQ332388**	phase 3	Proteobacteria; Gammaproteo-bacteria; Pseu-domonadales	Pseudomo-nadaceae; Pseudomo-nas	*Pseudomonas sp.* NJ-61 16S rRNA gene, strain NJ-61	AM421982	99	Uncultured soil bacterium clone T7_7 16S ribo-somal RNA gene, partial sequence	FJ184352.1 GI:208341952	**99**
KLnbIso11 GQ332389	phase 3	Proteobacteria; Gammaproteo-bacteria; Pseu-domonadales	Pseudomo-nadaceae; Pseudomo-nas	*Pseudomonas putida strain* KT-ql-116 16S ribosomal RNA gene, partial sequence.	FJ611926	100	*Pseudomonas putida* strain KT-ql-116 16S ribosomal RNA gene, partial sequence	FJ611926.1	**100**

KLnbIso12 GQ332390	phase 3	Proteobacteria; Gammaproteo-bacteria; Pseu-domonadales;	Pseudomo-nadaceae; Pseudomo-nas	*Pseudomonas sp.* Pi 3-8 gene for 16S rRNA, partial sequence	AB365065	94	Uncultured bacte-rium clone E4 16S ribosomal RNA gene, partial sequence	FJ347716	**98**